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AGONIST PEPTIDE FROM A CYTOTOXIC T-LYMPHOCYTE EPITOPE OF HUMAN CARCINOEMBRYONIC ANTIGEN STIMULATES PRODUCTION OF TC1-TYPE CYTOKINES AND INCREASES TYROSINE PHOSPHORYLATION MORE EFFICIENTLY THAN COGNATE PEPTIDE

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The identification of an agonist peptide (YLSGADLNL, designated CAP1-6D) to an immunodominant cytotoxic T-lymphocyte (CTL) epitope (designated CAP1) of human carcinoembryonic antigen (CEA) has previously been reported. The agonist peptide harbors a single amino acid substitution at a non-MHC anchor residue and is proposed to exert its effects at the level of the T-cell receptor (TCR). The type and magnitude of cytokines produced by CAP1-reactive CTL upon stimulation with the agonist peptide, CAP1-6D, were compared to those obtained upon stimulation with the cognate CAP1 peptide. In addition, early events in the TCR signaling pathway were examined for differences in tyrosine phosphorylation. Upon stimulation with the agonist peptide CAP1-6D, several different CEA-specific CTL lines exhibited a marked shift in the peptide dose response, which resulted in as much as a 1,000-fold increase in the levels of GM-CSF and γ-IFN produced as compared with the use of the CAP1 peptide. However, levels of IL-4 and IL-10, which are associated with anti-inflammatory effects, were very low or nonexistent. The cytokine profile of CAP1- and CAP1-6D-specific CTL is consistent with a Tc1-type CTL. Consistent with these findings, CEA-specific CTL showed increased tyrosine phosphorylation of TCR signaling proteins ZAP-70 and TCR ζ chains in response to both peptides. However, when CAP1-6D was compared with the wild-type peptide, the increase in ZAP-70 phosphorylation was greater than the increase in ζ phosphorylation. CTL generated with the CAP1-6D agonist were shown capable of lysis of human carcinoma cells expressing native CEA. The ability to upregulate the production of GM-CSF, γ-IFN, TNFα and IL-2 with the agonist peptide, as compared with CAP1, may help in initiating or sustaining anti-tumor immune responses and thus potentially prove to be useful in the treatment of CEA-positive tumors. *Int. J. Cancer* 85:829–838, 2000.
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Tumor immunotherapy most likely requires the activation of several effector functions of anti-tumor T cells. One major problem in inducing and maintaining anti-tumor immune responses has been identifying tumor-specific antigens to be used as immunogens. The majority of antigens thus far identified in human cancers can be characterized as tumor-associated antigens (TAA). These antigens are not tumor specific. Instead, they are overexpressed in malignant cells as opposed to cells of normal tissues. Therefore, cancer immunity in humans may rest mostly on the development of an effective immune response mainly directed to self-molecules that are common to tumor and normal cells. One explanation for the poor immune response to TAA may be the problem inherent in breaking tolerance in the generation and propagation of TAA-specific T cells.

Human carcinoembryonic antigen (CEA) is a 180-kDa glycoprotein expressed on the majority of colon, rectal, stomach and pancreatic tumors, on 50% of breast carcinomas, on 70% of lung carcinomas, in fetal gut tissue and, at lower levels, on normal colonic epithelium (Gold and Freedman, 1965; Muraro *et al.*, 1995). Several groups have now demonstrated the generation of anti-CEA antibodies and CEA-specific T-cell proliferative responses after immunization with either an anti-idiotype to an anti-CEA monoclonal antibody (MAb) (Foon *et al.*, 1995), recombinant CEA protein (Fagerberg *et al.*, 1996) or recombinant vaccinia-CEA (rV-CEA) (Conry *et al.*, 1995). Evidence of a human cytotoxic T lymphocyte (CTL) response to CEA has also recently been reported in a study describing the generation of specific CTL, directed against a 9-mer peptide designated CAP1, from carcinoma patients immunized with rV-CEA (Tsang *et al.*, 1995). In other studies, CAP1-pulsed dendritic cells (DCs) have been used successfully to generate CAP1-specific CTL (Alters *et al.*, 1998; Morse *et al.*, 1999; Nair *et al.*, 1998). CAP1-specific CTL have also recently been generated from peripheral blood mononuclear cells (PBMC) from carcinoma patients immunized with the avipox recombinant expressing CEA (ALVAC-CEA) (Marshall *et al.*, 1999*a*).

The cytokines produced by a cell upon activation and differentiation dictate, to a large extent, the cell's effector functions and direct the type of immune response. Different cytokine profiles have been shown to initiate, propagate or regulate tissue-specific autoimmune injury (Chen *et al.*, 1994; Nicholson and Kuchroo, 1996). In experimental allergic encephalomyelitis (EAE), an autoimmune model for multiple sclerosis, T-helper type 1 (Th1) cells that secrete pro-inflammatory cytokines (IL-2, γ-IFN, TNFα) induce autoimmunity (Kuchroo *et al.*, 1993), whereas Th2 cells (secreting IL-4 and IL-10) protect against EAE (Chen *et al.*, 1994; Nicholson and Kuchroo, 1996). Similarly, the development and maintenance of a good anti-tumor immune response may require not only the activation of tumor-specific cytotoxic T cells, but also the production of cytokines involved in the recruitment and activation of lymphocytes.

Slight modifications of peptide antigens to produce altered peptide ligands (APL) have been shown to markedly affect the type of immune response generated. Peptides modified at positions predicted to contact the T-cell receptor (TCR) have been shown to mediate TCR antagonism, induce T-cell anergy, partially activate T-cell clones, affect T-cell differentiation, protect animals from autoimmune disease and induce secretion of cytokines not detected with cognate antigen (De Magistris *et al.*, 1992; Sloan-Lancaster *et al.*, 1993; Evavold and Allen, 1991; Nicholson *et al.*, 1995, 1997, 1998; Kuchroo *et al.*, 1994; Windhagen *et al.*, 1995; Vergelli *et al.*, 1996). Another type of peptide modification is designed to increase major histocompatibility complex (MHC) binding. An example of a synthetic tumor-antigen peptide that has been modified at MHC anchor residues to improve binding to human leukocyte antigen (HLA)-A2 is the melanoma gp100 antigen (Rosenberg *et al.*, 1998).

It has been shown that a CTL epitope from human CEA can be modified to become more immunogenic by changing 1 amino acid

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residue at a position predicted to contact the TCR. Modification of position 6 of the CAP1 peptide from asparagine to aspartic acid was shown to induce a dramatic shift in the concentration of peptide required to give half-maximal CTL responses (Zaremba *et al.*, 1997). This enhanced CTL recognition was not due to better binding of the peptide to HLA-A2.1; instead, the enhanced CTL recognition has been proposed to act at the TCR level. T-cell lines generated from the CAP1-6D agonist are able to lyse human carcinoma cells expressing CEA (Zaremba *et al.*, 1997). The present report identifies responses of CAP1-specific human T cells to the native and modified peptide. Described here is a profile of cytokine production of CEA-specific CTL upon stimulation with either the original CAP1 epitope or the enhancer agonist peptide, CAP1-6D. When stimulated with the enhancer agonist peptide, tumor-specific CTL produced much higher amounts of GM-CSF, γ-IFN, TNFα and IL-2 than when stimulated with the CAP1 peptide. In addition, the pattern of tyrosine phosphorylation of proteins in the TCR signaling pathway suggests a qualitative difference between the response to native CAP1 and the agonist. When activated with agonist, the increase in ZAP-70 phosphorylation substantially exceeds the increase in ζ chain phosphorylation. The ability of the enhancer agonist peptide to induce high-level production of cytokines known to promote inflammatory responses and to alter TCR signaling may be crucial in establishing and maintaining a vigorous anti-tumor immune response *in vivo*.

MATERIAL AND METHODS

T-cell lines

PBMC were isolated from heparinized blood from healthy HLA-A2-positive individuals, from a patient (designated Vac8) with metastatic colon carcinoma who was enrolled in a phase I trial using rV-CEA (Tsang *et al.*, 1995) and from a patient (15) enrolled in a phase I trial who was immunized once with rV-CEA and then 3 times with ALVAC-CEA (Marshall *et al.*, 1999*b*). PBMC were separated using lymphocyte separation medium gradient (Organon Teknika, Durham, NC) as described (Boyum, 1968). All experiments involving patient materials were conducted according to NIH guidelines. Written, informed consent was obtained from all individuals.

T-N2 was generated from PBMC of a healthy HLA-A2-positive donor by *in vitro* stimulation (IVS) with the CAP1-6D peptide agonist (Zaremba *et al.*, 1997). The T-N2 CTL line has been maintained *in vitro* for more than 1 year by weekly stimulation with irradiated (20,000 rads) autologous Epstein-Barr virus (EBV)-transformed B cells pre-incubated with 3.12 μg/ml of CAP1-6D peptide at a ratio of 2.5:1, EBV-B cells to T cells. After 24 hr in culture, 10 U/ml human IL-2 and 0.1 ng/ml recombinant IL-12 (R&D Systems, Minneapolis, MN) were added. T-Vac8 CTL were generated from post-immunization PBMC from an HLA-A2-positive individual with advanced carcinoma who had received rV-CEA in a phase I trial. The T-Vac8 CTL line, the derivation of which has been described (Tsang *et al.*, 1997), has been maintained *in vitro* for 2 years by restimulation every 14 days with irradiated autologous PBMC (4,000 rads) or autologous EBV-B cells (20,000 rads) and 50 μg/ml CAP1 peptide. Cultures were fed with IL-2 (20 U/ml) every 3 days.

Cytotoxic assays

The SW1463 (colorectal carcinoma cell line, HLA-A1,2) and SK-mel (melanoma cell line) were purchased from ATCC (Manassas, VA). C1R-A2 cells are C1R cells that express a transfected genomic clone of HLA-A2.1. C1R-A2 cells were obtained from Dr. W.E. Biddison (National Institute of Neurologic Disorders and Stroke, NIH, Bethesda, MD). The cultures were free of Mycoplasma. C1R-A2 target cells or T2 cells were labeled with 200 μCi of ^{51}Cr for 1 hr and pulsed with or without the indicated peptides for an additional hour. After washing, target cells were counted and plated at 5,000 cells per well in 96-well round-bottom microtiter plates (Corning Costar, Cambridge, MA). T cells were added at various E:T ratios and incubated at 37°C in 5% CO_2 for 4 hr. The supernatant was harvested for gamma counting with the use of harvester frames (Skatron, Sterling, VA). All determinations were performed in triplicate, and specific lysis was calculated as: percent lysis = (experimental release − spontaneous release/ maximum release − spontaneous release) × 100.

$$\% \text{ lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Spontaneous release was obtained by omitting the T cells, and maximum release was achieved by adding 1% Triton X-100.

Cytokine assays

Cytokine production by the T-N2 and T-Vac8 (IVS-22) CTL lines was determined by stimulating 1×10^5 T cells with 1×10^5 irradiated (20,000 rads) autologous EBV-B or T2 cells with or without CAP1 or CAP1-6D at a wide range of peptide concentrations (0.00002–20 μg/ml). Cells were cultured in 48-well plates (Corning Costar) in RPMI 1640 with 10% human serum, 2 mM glutamine, and 10 μg/ml gentamycin in a total volume of 1 ml. Supernatant was harvested 24 and 48 hr later and stored at −20°C for cytokine analysis. Cytokine concentrations were detected from straight culture supernatant and 1:10 dilutions by specific capture ELISA according to the manufacturer's instructions (Endogen, Woburn, MA). The sensitivity of the ELISA was <2 pg/ml for GM-CSF, γ-IFN and IL-4; <5 pg/ml TNFα; and <3 pg/ml IL-10. The HLA-A2.1-binding Tax 119 peptide was used as an irrelevant control peptide. Parallel cultures of a Th0 T-helper cell line specific for the ras 5-17 peptide, known to secrete GM-CSF, γ-IFN, TNFα, IL-4 and IL-10 (Abrams *et al.*, 1997), were used as positive controls.

TCR usage determined by flow cytometry

Ficoll-purified T-N2 CTL (1×10^6) were stained with a panel of 25 anti-Vβ and 3 anti-Vα murine MAbs to human αβTCR variable regions. Cells were incubated with 10 μg/ml of purified FITC-labeled or PE-labeled antibodies for 45 min at 4°C. For dual-color flow cytometric analysis, the antibody pairs used were TCR Vβ5b-FITC (Vβ5.3 specificity, Endogen) and human CD8-PE (Becton Dickinson, San Jose, CA); TCR Vβ5b-FITC and TCRαβ-PE (Immunotech Coulter, Marseille, France); and TCRVβ5c-FITC (Vβ5.1 specificity, Endogen) and TCRαβ-PE. An antibody pair (IgG1-FITC and IgG1-PE, Becton Dickinson) was used as the isotype control. After 3 washes, cells were fixed with 1% paraformaldehyde, washed with a FACSFlow buffer and analyzed using a FACSort with the CellQuest program (Becton Dickinson).

TCR signaling

T2 cells (10^6) were pre-incubated for 2 hr either without peptide or with 10 μg/ml CAP1 or CAP1-6D, and then washed to remove unbound peptide. T-Vac8 cells were added (2×10^6), and the suspensions were microfuged briefly and incubated for 5 min in a 37°C water bath. Cells were washed in calcium/magnesium-free PBS, and pelleted and lysed for 30 min on ice with 0.1 ml cold, complete lysis buffer consisting of 1% NP-40 in 10 mM Tris-Cl, pH 7.2, 140 mM NaCl, 2 mM EDTA with 1 mM sodium orthovanadate, 5 mM iodoacetamide and Complete Protease Inhibitor (Boehringer Mannheim, Indianapolis, IN). After pelleting 5 min to remove insoluble material, lysates were immunoprecipitated with a rabbit anti-serum to ZAP-70 (generous gift of Dr. L. Samelson, National Institute of Child Health and Human Development, NIH, Bethesda, MD). Immunoprecipitates were washed, electrophoresed on 10% Tris-glycine gels, transferred to PVDF membranes, blocked and probed for phosphotyrosine with MAb 4G10 (Upstate Biotechnology, Lake Placid, NY). Antibody binding was revealed with peroxidase-conjugated anti-mouse Ig and chemiluminescence. To compare protein levels, the membrane was stripped and reprobed with rabbit anti-ZAP-70 or MAb to the TCR ζ chain (Zymed, San Francisco, CA). Densitrometric analysis was

performed on a Power Macintosh G3 computer (Apple Computer, Inc., Cupertino, CA) and a Hewlett Packard ScanJet IIcx (Hewlett Packard, Palo Alto, CA) using the public domain NIH Image program (developed at the NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/).

RESULTS

The generation of CTL lines from normal HLA-A2.1-positive PBMC utilizing the CAP1-6D agonist peptide has been described (Zaremba *et al.*, 1997). One such line, termed T-N2, results in lysis of peptide-pulsed C1R-A2 targets with at least 100-fold lower peptide concentrations when using the CAP1-6D peptide as compared with the CAP1 peptide (Fig. 1a). Similarly, T-Vac8 CTL generated from PBMC of a clinical trial patient immunized with rV-CEA and stimulated *in vitro* with CAP1 also lysed CAP1-6D-pulsed target cells much more efficiently than target cells pulsed with CAP1 (Fig. 1b). The lytic activity of T-N2 CTL against C1R-A2-pulsed target cells with a fixed peptide concentration of 0.5 μg/ml demonstrated higher lysis of CAP1-6D when compared with CAP1 peptide at various E:T ratios (Fig. 2).

Based on the prediction that the agonist peptide acts at the level of the TCR, the principle that other T-cell functions associated with TCR triggering would also be affected was postulated. A determination was sought as to whether the agonist effect was limited to the cytolytic function of the CTL or whether other parameters involved in the expansion and maintenance of a T-cell response would also be affected. Specifically, the cytokine production of T-N2 CTL was compared after stimulation with either the CAP1 or the CAP1-6D peptide. T-N2 CTL were stimulated with either autologous EBV-B cells or with T2 cells as antigen-presenting cells (APC) in the presence of various concentrations of CAP1 or the CAP1-6D peptide. GM-CSF production was detected after 24 hr of culture. T-N2 CTL produce GM-CSF when stimulated with the CAP1 peptide in a dose-dependent manner (Fig. 3). Stimulation of T-N2 CTL with the agonist CAP1-6D peptide required 50–100-fold less peptide concentration to achieve half-maximal production of GM-CSF. The T-N2 CTL line produces 500 pg/ml of GM-CSF after 24 hr of stimulation with 20 μg/ml of the CAP1 peptide. The same level of GM-CSF production was achieved with 0.2 μg/ml of the CAP1-6D peptide. After 48 hr of culture, half-maximal levels of GM-CSF were also detected with a 50-fold lower concentration of the CAP1-6D peptide as compared to CAP1. Moreover, using a peptide concentration of 2 μg/ml at 48 hr, 250 pg/ml of GM-CSF were produced when employing CAP1, whereas more than 600 pg/ml were produced employing the CAP1-6D peptide.

The T-N2 CTL line also produces γ-IFN in a peptide dose-response manner. Maximal production of 600 pg/ml was observed at the highest dose of CAP1 peptide used for stimulation (20 μg/ml) after 24 hr of culture. Stimulation with CAP1-6D at 100-fold (0.2 μg/ml) lower peptide concentration resulted in similar levels of γ-IFN (600 pg/ml) after 24 hr of culture (Fig. 4). Levels of γ-IFN produced upon stimulation with 0.2 μg/ml of CAP1-6D peptide start to drop off after 48 hr of culture. However, there was still about a 100-fold difference in the amount of CAP1-6D peptide required for the production of 400 pg/ml of γ-IFN as compared with the amount of CAP1 required.

The enhanced responses to the CAP1-6D peptide as compared with CAP1 may be due to the reactivity of different T-cell clones present in the T-N2 CTL line. To determine the heterogeneity of TCR usage in the T-N2 CTL line, Ficoll-purified T-N2 CTL were stained at the end of their stimulation cycle with a panel of 25 anti-TCR Vβ and 3 anti-Vα MAb. The results showed that 92% of the cells express Vβ5.3 and 12% express Vβ3.1. Dual-color analysis of T-N2 CTL showed that 98% of the selected population expressed both CD8 and TCRVβ5.3 (Fig. 5*a*), and 93% expressed both anti-TCRαβ and TCRVβ5.3 (Fig. 5*b*).

To determine that this pattern of more efficient stimulation with the agonist peptide was not due to the artificial selection of the T-N2 CTL line bearing a TCR that recognizes CAP1-6D better than CAP1, the cytokine profile of another CTL line (T-Vac8) was examined. The T-Vac8 CTL line, derived from a patient immu-

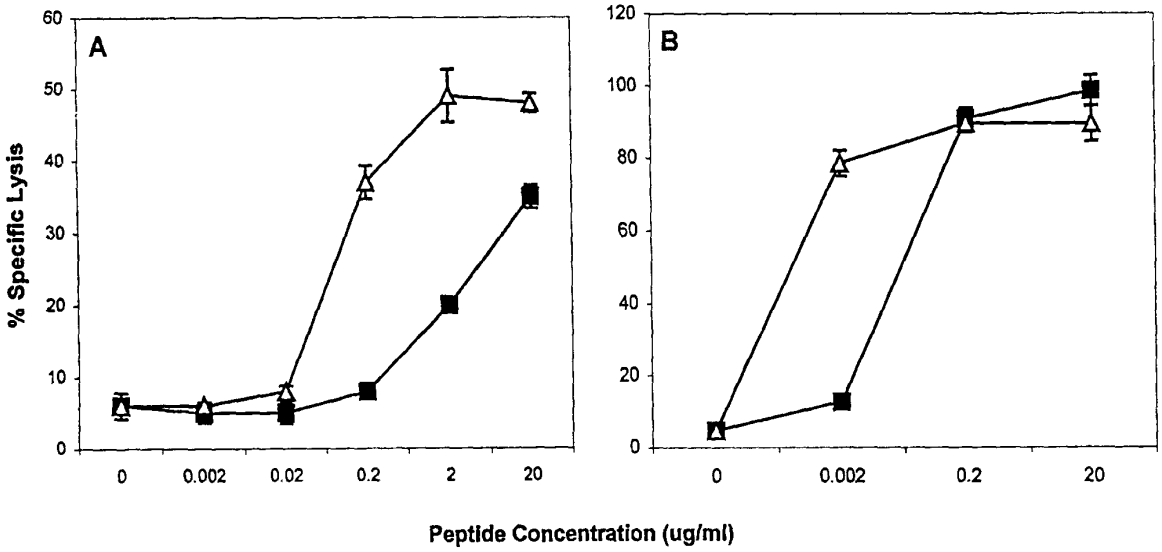

FIGURE 1 – CEA-specific CTL (T-N2 and T-Vac8) lyse T2 cells pulsed with the CAP1-6D agonist (open symbols) more efficiently than CAP1-pulsed (closed symbols) targets. T-N2 (IVS-12) (*a*) and T-Vac8 (IVS-25) (*b*) were cultured with ^{51}Cr-labeled T2 targets (5,000 cells per well) pulsed with the indicated peptide concentrations at an E:T ratio of 30:1 for T-N2 and 5:1 for T-Vac8. Peptide pulsing was done by incubating 3×10^5 T2 cells with or without the indicated peptide concentration in 0.5 ml of serum-free complete medium for 2 hr at 37°C. ^{51}Cr-release was determined after 4 hr of culture in a gamma counter. Results are representative of 3 independent experiments. Values determined from triplicate cultures are expressed as the mean ± SEM.

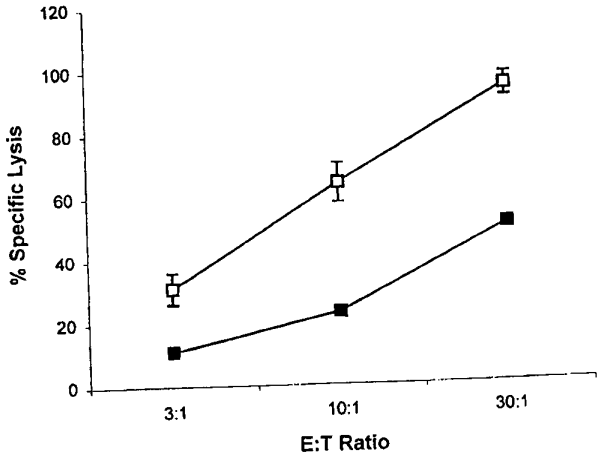

FIGURE 2 – Enhanced CTL lysis of the CAP1-6D agonist *vs.* CAP1-pulsed C1R-A2 targets. ^{51}Cr-labeled C1R-A2 targets (5,000 cells per well) were pulsed as described in Figure 1 with CAP1 (closed symbols) or CAP1-6D agonist (open symbols) at a fixed peptide concentration (0.5 μg/ml). Effector T-N2 CTL (IVS-14) were added at the indicated E:T ratios. ^{51}Cr-release was determined after 4 hr of culture in a gamma counter. Data are expressed as the mean ± SEM of triplicate cultures.

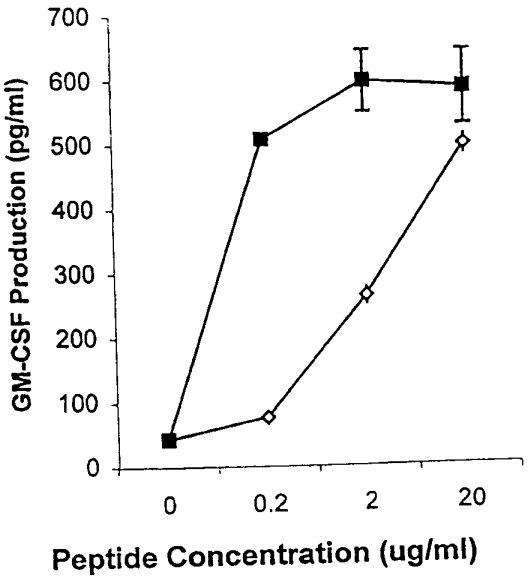

FIGURE 3 – GM-CSF production by CEA-specific CTL stimulated with either CAP1 (open symbols) or CAP1-6D (closed symbols) peptide; 3×10^5 T-N2 CTL were cultured with 3×10^5 T2 cells and the indicated peptide concentration in 24-well plates in a total volume of 2 ml.

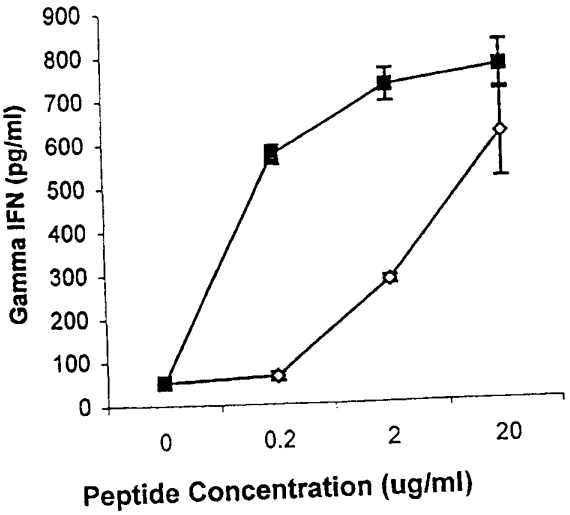

FIGURE 4 – γ-IFN production by CEA-specific CTL stimulated with either CAP1 (open symbols) or CAP1-6D (closed symbols) peptide. T-N2 CTL were cultured as described in Figure 3. γ-IFN production was determined using the Endogen ELISA kit. Values are expressed as the mean ± SEM of duplicate cultures.

nized with rV-CEA, has been cultured *in vitro* for 2 years in the presence of the CAP1 peptide. The T-Vac8 CTL line exhibits a limited number of TCR as determined by PCR analysis (Tsang *et al.*, 1997). After 20 IVS cycles, more than 90% of the total T-Vac8 population expressed Vβ1, 3, 4, 5 and 7 (Tsang *et al.*, 1997).

The amounts of GM-CSF produced by the T-Vac8 CTL line upon stimulation with its cognate antigen, CAP1, follow a peptide dose response (Fig. 6). Stimulation with 0.02 μg/ml of peptide results in the production of approximately 500 pg/ml of GM-CSF. The T-Vac8 CTL line produces maximal levels of GM-CSF (5,000 pg/ml) when stimulated with 2 μg/ml of cognate CAP1 peptide. Stimulation of T-Vac8 CTL with concentrations as low as 8 ng/ml of the agonist CAP1-6D peptide results in comparable levels of GM-CSF (5,000 pg/ml) production. This is a 250-fold difference in peptide concentration to achieve equal GM-CSF levels (Fig. 6). GM-CSF production increases to approximately 9,000 pg/ml when stimulated with 0.2 μg/ml of the agonist CAP1-6D peptide.

T-Vac8 production of another inflammatory cytokine, γ-IFN, was even more marked. Maximal γ-IFN production of 8,000 pg/ml was obtained by stimulating T-Vac8 CTL with 0.2 μg/ml of the CAP1-6D agonist peptide (Fig. 7). Concentrations as low as 0.02 μg/ml of the agonist peptide resulted in half-maximal production of γ-IFN. However, stimulation of the T-Vac8 CTL line with as much as 20 μg/ml of its cognate CAP1 peptide resulted in γ-IFN levels lower (3,000 pg/ml) than those achieved with 0.02 μg/ml of CAP1-6D (a 1,000-fold difference in peptide concentration).

The production of TNFα by CEA-specific CTL was also examined, based on the known cytolytic properties of this cytokine. Stimulation of T-Vac8 CTL with high concentrations of the CAP1 peptide (20 μg/ml) resulted in the production of TNFα (Fig. 8). T-Vac8 CTL are capable of producing higher levels of TNFα, however, when stimulated with a 100-fold lower concentration of the CAP1-6D agonist peptide.

Studies were undertaken to determine whether peptide stimulation of the T-N2 and T-Vac8 CTL lines also resulted in the production of cytokines known to inhibit immune responses; if so, the studies were meant to determine if the agonist peptide had any effect. Table I shows that stimulation of either T-N2 or T-Vac8 CTL with high concentrations of CAP1 or CAP1-6D failed to produce the inhibitory cytokine, IL-10. In contrast, a Th0 T-helper line specific for the ras 5-17 peptide (Abrams *et al.*, 1997) produces high levels of IL-10 upon antigenic stimulation. T-N2 CTL did not produce IL-4, which is also known to inhibit cell-mediated immune responses such as delayed-type hypersensitivity. However, stimulation of T-Vac8 CTL with CAP1 or CAP1-6D peptides resulted in low levels of IL-4 production. The cytokine profile of the T-N2 and T-Vac8 CTL lines is consistent with the CTL of the Tc1 type. Stimulation with the agonist peptide appears to enhance

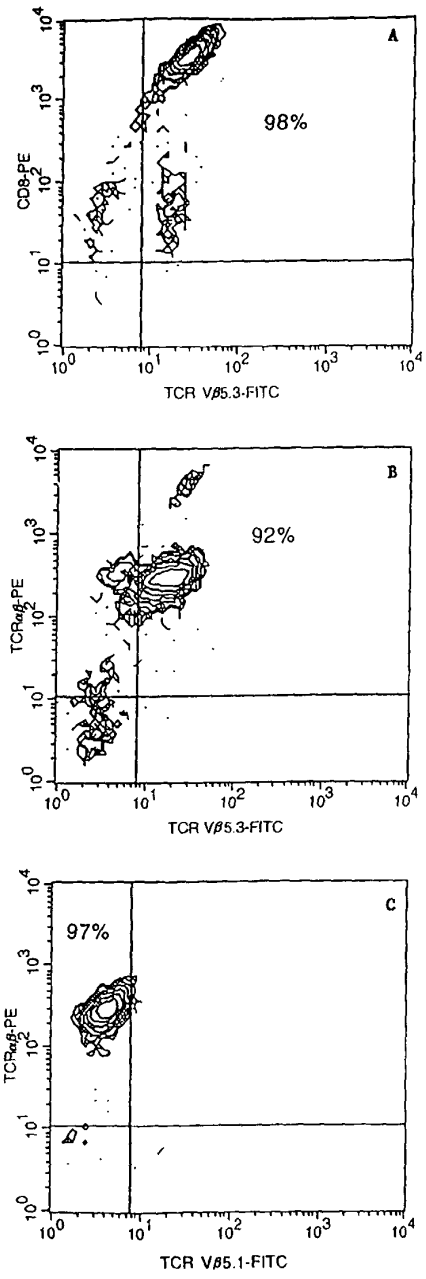

FIGURE 5 – CEA-specific CTL TCR Vβ usage. Ficoll-purified T-N2 CTL (5×10^5) were dually stained with 20 μl of the indicated, directly-labeled antibodies. Anti-TCR Vβ5.3-FITC and anti-CD8-PE (*a*); anti-TCR Vβ5.3-FITC and anti-TCR pan αβ-PE (*b*); and anti-TCR Vβ5.1-FITC and anti-TCR αβ-PE (*c*). IgG1-FITC and IgG1-PE antibodies were used as isotype controls. Fluorescence was analyzed on a Becton Dickinson FACSort.

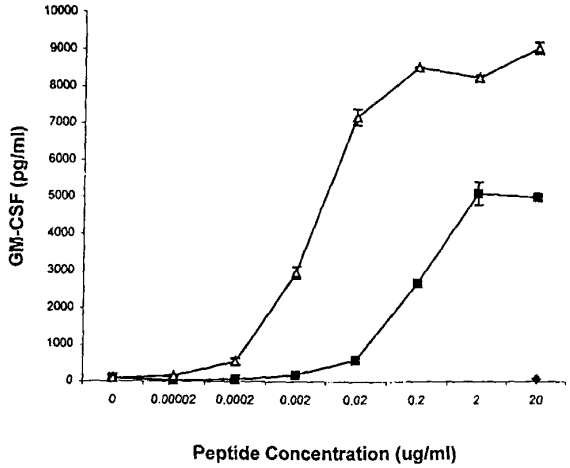

FIGURE 6 – CEA-specific T-Vac8 CTL produce higher levels of GM-CSF when stimulated with the CAP1-6D agonist as compared with the cognate CAP1 peptide; 1×10^5 T-Vac8 CTL (IVS-22) were cultured with 1×10^5 T2 cells with either CAP1 (closed square) or CAP1-6D (open triangle) at the indicated peptide concentrations in a total volume of 1 ml in each well of a 48-well plate. The Tax 119 peptide (closed diamond) was used as a control.

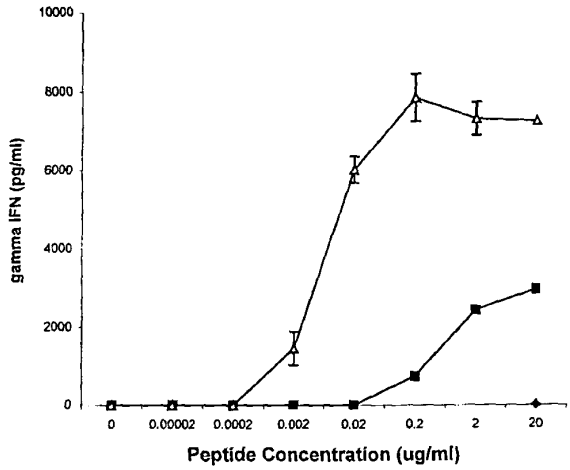

FIGURE 7 – CEA-specific T-Vac8 CTL produce higher levels of γ-IFN when stimulated with the CAP1-6D (open triangle) agonist as compared with the native CAP1 peptide (closed square). T-Vac8 CTL were cultured as described in Figure 6. Duplicate samples were assayed by specific capture ELISA (Endogen). Values are expressed as the mean ± SEM of duplicate cultures. The Tax 119 peptide (closed diamond) was used as a control.

production of cytokines known to augment inflammatory responses.

Studies were then conducted using PBMC from a patient (15) with metastatic carcinoma who had been vaccinated with rV-CEA and then boosted 2 times with ALVAC-CEA (Marshall *et al.*, 1999*b*). T cells from this patient were purified and stimulated using autologous DC pulsed with either the CAP1 peptide (T-cell line designated T-15-CAP1) or the agonist CAP1-6D (T-cell line designated T-15-CAP1-6D). IFN-γ production was measured at each IVS cycle. Although moderate increases in IFN-γ are seen at IVS-1 and IVS-2 using CAP1-6D, significant differences using CAP1-6D *vs.* CAP1 are seen at IVS-3 (Table II). These 2 T-cell lines were also analyzed for IL-2 production as a consequence of stimulation with the CAP1 or the CAP1-6D peptide. The T-15-CAP1-6D line showed enhanced IL-2 production over the T-15-CAP1 line when stimulated with either the CAP1 or the CAP1-6D

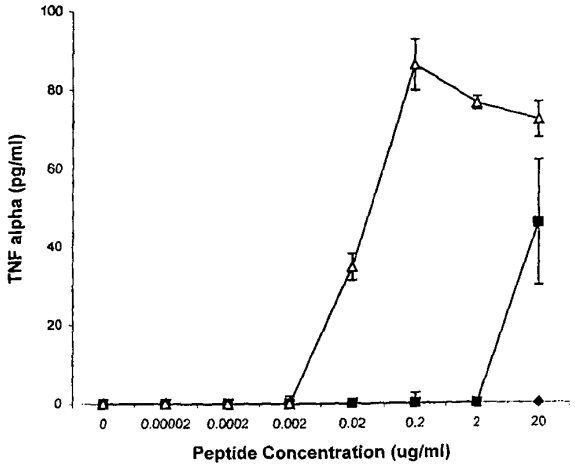

FIGURE 8 – Stimulation of CEA-specific T-Vac8 CTL with the CAP1-6D agonist enhances production of TNFα. T-Vac8 CTL were cultured as described in Figure 6. Cells were stimulated with either CAP1-6D (open triangle), CAP1 (closed square) or the control Tax 119 peptide (closed diamond). Duplicate samples were assayed by specific capture ELISA (Endogen). Values are expressed as the mean ± SEM of duplicate cultures.

TABLE I – STIMULATION OF CAP1 AND CAP1-6D-SPECIFIC CTL RESULTS IN LITTLE OR NO PRODUCTION OF TC2-TYPE CYTOKINES[1]

CTL	Peptide or antibody	μg/ml	Cytokine (pg/ml)	
			IL-4	IL-10
T-Vac8	None		1	0
	Tax 119	20	0	0
	Anti-CD3	5	57	0
	CAP1	20	63	0
		2	32	0
		0.2	25	0
	CAP1-6D	20	90	0
		2	95	0
		0.2	100	0
T-N2	CAP1	20	0	0
		2	0	0
		0.2	0	0
	CAP1-6D	20	0	0
		2	0	0
		0.2	0	0
5-17 V12	*ras* 5-17 (V12)	10	1,172	1,223
5-17 V12	*ras* 5-17 (G12)	10	18	0

[1]CTL were cultured with T2 cells and the indicated peptide concentration or treatment. Supernatant was obtained after 24 hr of culture and assayed for cytokine production in duplicate using an Endogen ELISA kit. The 5-17 V12 T-helper line is specific for the *ras* 5-17 (V12) peptide and does not recognize *ras* 5-17 (G12).

peptide (Table III). The T-cell lines derived from this patient after stimulation with CAP1 or CAP1-6D (IVS-4) were then analyzed for their ability to lyse peptide-pulsed cells as well as human carcinoma cells expressing CEA (Table IV). The cell line derived by stimulation with CAP1-6D showed statistically significant increases in lysis when compared with the cell line derived from CAP1 stimulation when the target cells were (a) C1R-A2 cells pulsed with CAP1, (b) C1R-A2 cells pulsed with CAP1-6D and (c) SW1463 colon carcinoma cells that are $A2^+$ and express CEA. No increase in lysis was seen for the T-15-CAP1-6D line *vs.* the T-15-CAP1 line when C1R-A2 cells alone or SK-mel melanoma cells ($A2^+$ and CEA^-) were used as targets. In additional experiments (Table V), the CTL line T-Vac8, obtained from a patient immunized with rV-CEA (Tsang *et al.*, 1995) and then stimulated

TABLE II – SPECIFIC RELEASE OF γ-IFN BY T CELLS IN RESPONSE TO CAP1 OR CAP1-6D-PULSED DENDRITIC CELLS (DC)[1]

Treatment	*In vitro* stimulation cycle		
	1	2	3
T cells + DC + CAP1	17.9[2] (3.2)	75.1 (4.8)	124.9 (11.4)
T cells + DC + CAP1-6D	37.5 (6.9)	187.5 (12.4)	464.7 (18.3)[3]
T cells + DC	12.4 (9.2)	8.3 (1.5)	ND

[1]T cells (designated T-15) were derived from the PBMC of a patient with metastatic pelvic carcinoma who had been vaccinated with rV-CEA and then boosted with ALVAC-CEA. Autologous DC as APC were infected with rF-B7-1 at a multiplicity of infection (MOI) of 60:1 for 2 hr. The infected DC were suspended in 10 ml of fresh, warm RPMI-1640 complete medium containing 50 ng/ml of rh-GM-CSF and 0.5 ng/ml of rhIL-4, cultured for 24 hr, and subsequently used as stimulators. T cells (0.5×10^6/ml) were stimulated with irradiated autologous DC pulsed with 20 μg/ml of CAP1 or CAP1-6D. Twenty-four-hour culture supernatants were collected and screened for γ-IFN using an ELISA kit (R&D Systems) [ND, not done.]–[2]Results are expressed in pg/ml γ-IFN of culture supernatant (standard deviation in parentheses).–[3]Statistically significant ($p < 0.01$, two-tailed *t*-test) for CAP1-6D *vs.* CAP1.

TABLE III – RELEASE OF IL-2 BY T-CELL CULTURES ESTABLISHED WITH CAP1 OR AGONIST CAP1-6D PEPTIDE[1]

Treatment	T-15-CAP1	T-15-CAP1-6D
CAP1 + APC	192.0[2] (2.4)	453.1 (4.8)[3]
CAP1	<40	<40
CAP1-6D + APC	384 (4.9)	510.6 (3.2)[3]
CAP1-6D	<40	<40
APC	<40	<40
None	<40	<40

[1]T cells (from patient 15) were stimulated with CAP1 or CAP1-6D (10 μg/ml) peptide-pulsed autologous EBV-transformed B cells (APC). Twenty-four hour culture supernatants were collected and screened for the secretion of IL-2 using an ELISA kit (Endogen).–[2]Results are expressed in pg/ml IL-2 (SD) of cell culture at a concentration of 1×10^6 T cells/ml. T cells: APC were used at a ratio of 1:3. T-cell lines were used at IVS-5.–[3]Statistically significant ($p < 0.01$, two-tailed *t*-test) for T-15-CAP1-6D *vs.* T-15-CAP1.

TABLE IV – CYTOTOXICITY OF T-CELL LINES ESTABLISHED WITH CAP1 AND CAP1-6D PEPTIDES FROM A PATIENT POST-VACCINATION WITH RECOMBINANT CEA VACCINES[1]

Target cells	T-15-CAP1	T-15-CAP1-6D
C1R-A2	12.5[2] (1.5)	11.3 (2.28)
C1R-A2 + CAP1	19.9 (0.42)	26.4 (1.54)[3]
C1R-A2 + CAP1-6D	28.7 (3.22)	42.2 (1.18)[3]
SW1463	13.7 (1.57)	25.1 (2.51)[3]
SK-mel	2.8 (0.48)	3.1 (1.90)

[1]T cells were derived from the PBMC of a patient with metastatic pelvic carcinoma who had been vaccinated with rV-CEA and then boosted with ALVAC-CEA. T-15-CAP1 and T-15-CAP1-6D are T-cell lines established from this patient (#15) using CAP1 or CAP1-6D peptide-pulsed dendritic cells, respectively. A 12-hr ^{111}In-release assay was performed. CAP1 and CAP1-6D peptides were used at a concentration of 10 μg/ml. Results are expressed as percentage of specific lysis (SD) at an E:T ratio of 25:1. T-cell cultures were used at IVS-4.–[2]Percent cytotoxicity (SD in parentheses).–[3]Statistically significant lysis ($p < 0.01$, two-tailed *t*-test) using CTL T-15-CAP1-6D *vs.* CTL T-15-CAP1.

with CAP1-6D, retained the ability to lyse SW1463 colon carcinoma cells ($A2^+$, CEA^+), but not SK-mel melanoma cells ($A2^+$, CEA^-).

T-Vac8 CTL and CTL derived from a second vaccinated patient showed stronger responses to CAP1-6D than to CAP1 when examined for lytic ability and production of cytokines. For T-Vac8, the dose-response curve for each of these functions shifted to more

TABLE V – ABILITY OF A T-CELL LINE (T-VAC8) DERIVED BY STIMULATION WITH AGONIST CAP1-6D TO LYSE TUMOR CELLS EXPRESSING NATIVE CEA OR CELLS PULSED WITH CAP1[1]

Target cells	Percent of specific lysis (SD)
C1R-A2	8.39 (1.08)
C1R-A2 + CAP1	22.9 (6.35)[2]
C1R-A2 + CAP1-6D	46.6 (4.20)[2]
SW1463	27.1 (2.11)[2]
SK-mel	2.1 (0.57)

[1]The T-Vac8 T-cell line was established from a patient with metastatic colon carcinoma vaccinated with rV-CEA (Tsang *et al.*, 1997). The protocol described by Tsang *et al.* (1995) with CAP1-6D peptide was used to generate the T-cell line. A 12-hr ^{111}In-release assay was performed. CAP1 and CAP1-6D peptides were used at a concentration of 10 μg/ml. Results are expressed as a percentage of specific lysis (SD) at an E:T ratio of 25:1. T-cell cultures were used at IVS-12.– [2]Statistically significant lysis ($p < 0.01$, two-tailed t test) using C1R-A2 plus CAP1-6D, or C1R-A2 plus CAP1 *vs.* C1R-A2 and SW1463 *vs.* SK-mel.

sensitive half-maximum values by differing orders of magnitude. In addition, the maximum production of GM-CSF and γ-IFN obtained at optimal peptide concentration was at least 2-fold greater with the agonist. This suggested that intracellular signals delivered through the TCR might differ qualitatively when comparing the 2 peptides. Figure 9a shows that both peptides at 10 μg/ml produce maximum and equivalent levels of lysis, consistent with the previous results (Fig. 1b). Nonetheless, inspection of the tyrosine phosphorylation of cellular proteins reveals differences between the response to the 2 peptides at this equipotent stimulation. As expected, ZAP-70 is phosphorylated in T-Vac8 CTL stimulated with CAP1 or CAP1-6D, but not in the absence of peptide (Fig. 9b, top). However, phosphotyrosine levels are clearly stronger with CAP1-6D than with CAP1. This is not due to differences in protein loading; in fact, protein levels appear equal in the 2 lanes (Fig. 9b, bottom) when the gel is stripped and reprobed with ZAP-70 antibody.

T-cell activation induces tyrosine phosphorylation of TCR ζ chains followed by association with ZAP-70 (Weiss and Littman, 1994; Wange and Samelson, 1996). Figure 9c shows the amount and phosphorylation state of ζ chains that jointly immune precipitated with ZAP-70. Only trace amounts co-precipitated in the absence of peptide (Fig. 9c, bottom). But when stimulated with CAP1 or CAP1-6D, the amount of ζ protein recruited to the complex increased significantly. More ζ protein was present when cells were stimulated with CAP1-6D than with CAP1, and there appeared to be relatively greater accumulation of higher m.w. isoforms. Phosphorylation of ζ chains was also stimulation dependent and greater with CAP1-6D than with CAP1 (Fig. 9c, top). However, the increased phosphotyrosine levels seen with the agonist may be due to greater recruitment of ζ protein to the ZAP-70 complexes. Densitrometric measurements (Fig. 9d) confirmed that the percent increase in ZAP-70 phosphorylation was greater than the increase in ζ phosphorylation when the agonist response was compared to the wild-type activation. This disparity between the increase in ZAP-70 and ζ phosphorylation was seen in additional experiments at suboptimal peptide concentrations (data not shown). The demonstration that the 2 phosphorylation events do not increase in parallel indicates a qualitative difference between the CAP1 and agonist response.

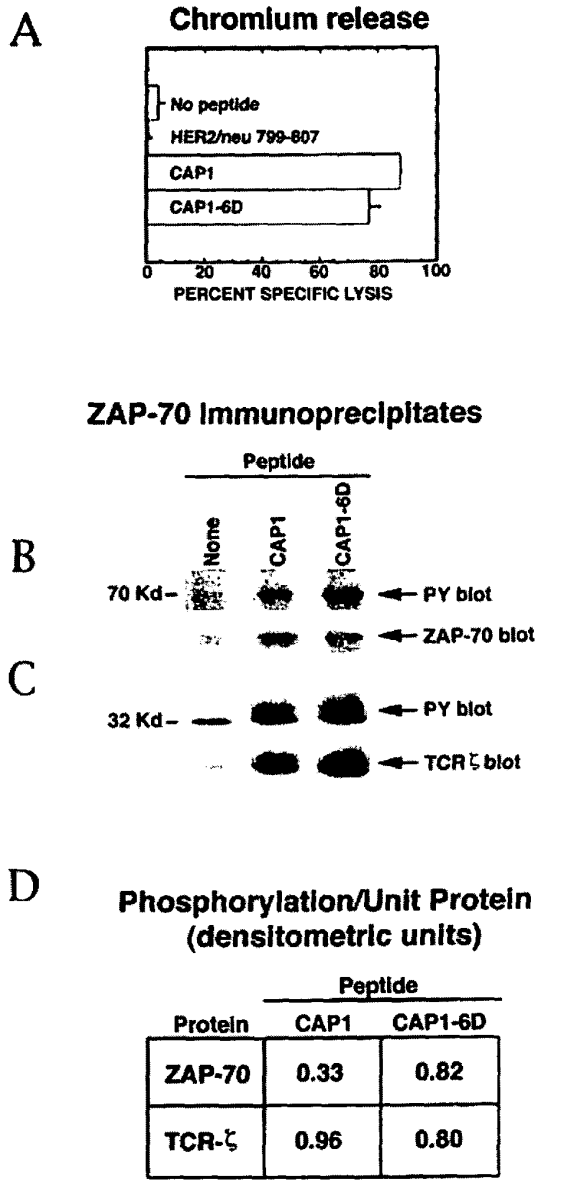

FIGURE 9 – T-Vac8 CTL generate stronger intracellular signaling with CAP1-6D than with CAP1. (*a*) Chromium-labeled T2 cells were pulsed without peptide or with 10 μg/ml peptides HER2/neu 799-807 (negative control), CAP1 or CAP1-6D. Rested T-Vac8 CTL were added at a 2.5:1 E:T ratio, and isotope release was measured after 4 hr. (*b,c*) Unlabeled T2 (10^6) without or with 10 μg/ml peptide were incubated for 5 min at 37°C with rested T-Vac8 (2×10^6), lysed, and immunoprecipitated with anti-ZAP-70 Ab. Washed precipitates were electrophoresed, transferred to PVDF and probed with (*b*, top) antibody to phosphotyrosine. (*b*, bottom) Membrane was stripped and reprobed with anti-ZAP-70 Ab. (*c*, top) Brief re-exposure of (*b*, top) reveals phosphotyrosine state of TCR ζ chains that co-precipitated with ZAP-70. (*c*, bottom) Membrane was reprobed with anti-ζ Ab. (*d*) Densitometric analysis of *b* and *c* (arbitrary units) shows that when comparing CAP1-6D to CAP1, the increase in ZAP-70 phosphorylation exceeds the increase in ζ chain phosphorylation.

DISCUSSION

This study shows that stimulation of CTL specific for a 9-mer epitope of human CEA antigen, designated CAP1 (Zaremba *et al.*, 1997), with a modified peptide agonist dramatically shifts the peptide concentration that results in maximal production of cytokines associated with inflammatory responses (i.e., GM-CSF, γ-IFN and TNFα). The CAP1 peptide, originally selected based on its strong binding to HLA-A2 and its lack of identity to other members of the CEA gene family, has been defined as an immunodominant epitope of CEA (Tsang *et al.*, 1995; Alters *et al.*,

1998; Morse *et al.*, 1999; Nair *et al.*, 1998; Zaremba *et al.*, 1997). CTL have been generated from rV-CEA-immunized PBMC using the CAP1 peptide to stimulate PBMC *in vitro*. Such CAP1-specific CTL have been shown to lyse CEA-positive tumors in an HLA-A2-restricted fashion. However, generating and expanding CAP1-specific CTL *in vitro* has proven to be difficult. The identification of an enhancer agonist CTL peptide has been reported in an attempt to improve the immunogenicity of the CAP1 peptide (Zaremba *et al.*, 1997). The enhancer agonist peptide was originally identified from a panel of single amino acid substitutions at non-MHC anchor positions predicted to contact the TCR. When using CAP1-specific CTL obtained from 2 independent rV-CEA-immunized PBMC as effector cells, the CAP1-6D agonist sensitized target cells for lysis far better than the cognate antigen CAP1 (Zaremba *et al.*, 1997). Although there is no direct evidence, it is unlikely that an asparagine to aspartic acid substitution occurs naturally in CEA according to the mechanism proposed by Skipper *et al.* (1996), since the Asn does not lie in the obligatory consensus N-glycosylation site.

Based on its enhanced cytolytic potential, the CAP1-6D agonist has become a candidate peptide for use as a vaccine (active immunotherapy) and for *ex vivo* expansion of CTL for adoptive transfer (passive immunotherapy) in clinical trials of HLA-A2-positive patients with solid tumors expressing CEA. A variety of tumors known to express CEA antigen or epitopes would be likely candidates for a CAP1-6D agonist-based vaccine. These include tumors of the colon, stomach, lung, pancreas, endometrium and breast, as well as other types of cancer.

Activation of anti-tumor CTL is believed to be very important for tumor eradication. Presumably, cytokines released by tumor-specific CTL present at the site of the tumor may dictate the type of localized immune response that would follow. Because cytokines can have both stimulatory and inhibitory effects on lymphocytes, it was important to determine the nature and magnitude of cytokines produced by CAP1-specific CTL upon cognate and agonist peptide activation. For instance, IL-4 and IL-10 normally produced by Th2 cells are known to inhibit macrophage activation. On the other hand, γ-IFN is known to promote T-cell differentiation to the Th1 phenotype, promote maturation of $CD8^+$ CTL, activate macrophages and NK cell activity, upregulate MHC expression and increase the expression of costimulatory molecules. Activated macrophages also produce TNF, which in itself may be involved in tumor eradication. All of these parameters may play an important role in enhancing the immune response to tumors.

Cytokine production of the following CAP1-specific cytotoxic T-cell lines was examined: T-N2 derived from normal HLA-A2-positive PBMC by IVS with the CAP1-6D agonist peptide, and T-Vac8 derived from HLA-A2-positive PBMC from a patient immunized with rV-CEA and stimulated *in vitro* with the CAP1 peptide. Both CTL lines exhibit cytolytic activity against target cells pulsed with either the CAP1 peptide or the CAP1-6D agonist. However, lysis of CAP1-6D agonist-pulsed targets is 1–2 orders of magnitude higher than lysis of target cells pulsed with the CAP1 peptide. Cytokine production upon stimulation of CAP1-specific CTL with the CAP1-6D agonist also results in elevated production of GM-CSF and γ-IFN. Furthermore, stimulation of T-Vac8 CTL with the CAP1-6D peptide results in production of TNFα, even at a low peptide concentration. However, TNFα production was observed only at the highest peptide dose when the CAP1 peptide was used to stimulate T-Vac8 CTL. Stimulation of CAP1-specific CTL with either peptide resulted in little or no production of IL-4 and IL-10. Overall, the cytokines produced by T-N2 and T-Vac8 CTL are indicative of a Tc1 phenotype.

It is not surprising that the T-N2 CTL line derived from a healthy, non-immunized individual and maintained *in vitro* in the presence of the CAP1-6D agonist is more efficient at lysing target cells pulsed with the agonist peptide as compared with CAP1. However, T-N2 CTL also lyse CEA-positive, HLA-A2-positive tumor cells, which suggests that *in vitro* selection of a T-cell line using the agonist peptide is compatible with the recognition of the endogenous tumor antigen expressed by the tumor cell. It is perhaps more relevant that in experiments reported here, and in others shown previously (Zaremba *et al.*, 1997), 2 CTL lines obtained from 2 patients immunized with CEA vaccines and stimulated *in vitro* with the CAP1-6D agonist peptide lyse CEA-positive tumors in an HLA-A2-positive restricted manner.

Because anti-CAP1 CTL from several donors demonstrate agonist cross-reactivity, it is possible that CAP1-6D may be used to stimulate growth of CTL from numerous HLA-A2.1-positive individuals that may be used for passive immunotherapy and as a peptide-based vaccine for active immunotherapy. There is evidence of TCR heterogeneity in the recognition of modified peptide antigens. A wide range of different peptides has been shown to interact with a single TCR (Hemmer *et al.*, 1997). CTL exhibiting different patterns of TCR Vβ usage (T-Vac8, T-Vac24, T-N1 and T-N2) recognize the CAP1-6D agonist peptide far better than the native CAP1 epitope. These CTL are also capable of lysing tumor cells that express CEA. Thus, the ability of CAP1-6D to act as an agonist with T cells expressing different TCR magnifies its therapeutic potential. The difference in the quality or degree of the TCR signal delivered with the agonist peptide as opposed to the CAP1 peptide may stimulate the cell to produce higher levels of cytokines at very low peptide concentrations and may trigger a more effective cytolytic response.

The early description of T-cell activation by altered peptide ligands (Evavold and Allen, 1991) has been extended to various activated human $CD8^+$ CTL of clinical significance (Zaremba *et al.*, 1997; Hollsberg *et al.*, 1995; Dressel *et al.*, 1997; Rivoltini *et al.*, 1999). Substituted peptides, ranging from superagonists to weak agonists and partial agonists to antagonists, have been defined in functional and biochemical terms. Partial agonists are substituted peptides that selectively activate some, but not all, functions of T cells. Our present studies with different CTL demonstrate a number of differences in the responses to CAP1 and CAP1-6D. However, the biologically native CAP1 used to generate the CTL exhibits more features of a partial agonist, as compared with the CAP1-6D altered peptide ligand. Firstly, even at optimal peptide stimulation, CAP1 produces only half as much GM-CSF (Fig. 6) and γ-IFN (Fig. 7) as CAP1-6D. Secondly, the shifts in dose-response curves between CAP1 and CAP1-6D vary from function to function. The concentrations of CAP1 required for maximal activation are greater by factors of 100–200 for cytotoxicity (Zaremba *et al.*, 1997), 250 for GM-CSF and 2,000 for IFN. Thirdly, ZAP-70 phosphorylation, an early event in the TCR signaling pathway, is disproportionately higher with CAP1-6D activation than with CAP1. Significantly, with CAP1-6D, the percent increase in ZAP-70 phosphorylation is greater than that of ζ phosphorylation.

TCR signaling of mature T cells with antigens and APL also differs in that additional, direct isoforms of the TCR ζ chain are phosphorylated with full agonists. Detailed studies with full or superagonist stimulation (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1995; Rabinowitz *et al.*, 1996; Crowe *et al.*, 1998; Reis e Sousa *et al.*, 1996; Hemmer *et al.*, 1998; Loftus *et al.*, 1998) describe the appearance of a high m.w. isoform. This phosphoprotein is diminished under weak or partial agonist stimulation and is absent altogether when antagonized T cells are stimulated. Our ZAP-70 immunoprecipitates show increases in ζ phosphorylation and co-precipitation when CAP1-6D and CAP1 stimulation are compared. In particular, the phosphotyrosine signal with CAP1-6D is stronger in the higher m.w. isoform region, recalling the increased high/low isoform ratio seen in other systems (Hemmer *et al.*, 1998). In conclusion, a conservative interpretation of our findings is that along the continuum of stimulation responses, CAP1-6D stimulation exhibits more full-agonist features, whereas CAP1 stimulation exhibits more partial-agonist features.

As observed here, activation by CAP1-6D, an agonist peptide that has MHC-binding affinity similar to the cognate ligand CAP1,

can alter the magnitude and pattern of the T-cell response. Partial agonists have been defined as APL that deliver partial signals to the T cell (Evavold and Allen, 1991; Evavold *et al.*, 1993). Partial agonists either initiate the same functions as the cognate ligand but require higher concentrations of antigen to achieve the same effect (weak agonists) or elicit only a subset of the functions stimulated by the cognate ligand (partial agonists). Finally, superagonists hyperstimulate T-cell clones with respect to the cognate ligand (Hemmer *et al.*, 1997). The CAP1-6D agonist in the system reported here appears to act as a better antigen for CAP1-specific T-cell lines. The functional data suggest that the agonist peptide would be a more efficient immunogen in clinical trials of HLA-A2-positive patients with CEA-positive tumors.

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We claim:

1. A peptide comprising an agonist of a native sequence:

YLSGANLNL (Seq. ID No: 1)
| |
123456789

2. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 6 from Seq. ID No: 1.

3. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 7 from Seq. ID No: 1.

4. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 6 and position 7 from Seq. ID No: 1.

5. The peptide according to claim 1 containing YLSGADLNL (Seq. ID No: 2), YLSGADINL (Seq. ID No: 3), YLSGANINL (Seq. ID No: 4), YLSGACLNL (Seq. ID No: 5), or combination thereof.

6. A peptide consisting of the amino acid sequence YLSGADLNL (Seq. ID No: 2), YLSGADINL (Seq. ID No: 3), or YLSGANINL (Seq. ID No: 4), YLSGACLNL (Seq. ID No: 5).

7. A pharmaceutical composition comprising the peptide according to claim 1 and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition according to claim 7 further comprising an immunostimulatory molecule.

9. The pharmaceutical composition according to claim 8 wherein the immunostimulatory molecule is selected from the gproup consisting of IL-2, B7.1, B7.2, ICAM-1,

LFA-3, CD72, GM-CSF, TNFα, INFγ, IL-12, IL-6 and combinations thereof.

10. The pharmaceutical composition according to claim 7 further comprising an HLA class I molecule or a cell expressing an HLA class I molecule.

11. The pharmaceutical composition according to claim 7 further comprising a chemotherapeutic drug, antibiotic, antiviral drug, antifungal drug, or cyclophosphamide.

12. The pharmaceutical composition according to claim 7 further comprising an adjuvant.

13. The pharmaceutical composition according to claim 12 wherein the adjuvant is selected from the group consisting of alum, incomplete Freund's adjuvant, QS21, and Ribi Detox™.

14. A peptide-immunoglobulin conjugate comprising the peptide according to claim 1 and an immunoglobulin molecule.

15. The pharmaceutical composition according to claim 7 wherein the peptide is incorporated into a liposome.

16. A peptide-carrier molecule conjugate comprising the peptide according to claim 1 conjugated to a carrier molecule.

17. The peptide-carrier molecule conjugate according to claim 15 wherein the carrier molecule is selected from the group consisting of influenza peptide, tetanus toxoid, tetanus toxoid-CD4 epitope, Pseudomonas exotoxin A, poly-L-lysine, a lipid tail and an endoplasmic reticulum signal sequence.

18. A kit comprising the agonist peptide

according to claim 1 and a vector comprising a nucleic acid sequence encoding CEA.

19. The kit according to claim 18 further comprising an immunostimulatory molecule.

20. An isolated DNA comprising a nucleotide sequence encoding the peptide according to claim 1 or variants thereof.

21. An isolated DNA encoding a peptide containing Seq. ID No: 2, Seq. ID No: 3, Seq. ID No: 4, Seq. ID No: 5, or combinations thereof.

22. An isolated DNA comprising a nucleotide sequence of SEQ. ID No: 7 or 8.

23. A vector comprising the DNA of claims 20, 21 or 22.

24. The vector according to claim 23 wherein the vector is an E. coli plasmid, a Listeria vector, an orthopox virus, avipox virus, capripox virus, suipox virus, vaccinia virus, baculovirus, human adenovirus, SV40 or bovine papilloma virus.

25. The vector according to claims 23 or 24 further comprising a nucleotide sequence encoding at least one HLA class I molecule.

26. A host cell comprising the vector according to claim 23.

27. The host cell according to claim 26 wherein the host cell additionally expresses an HLA class I molecule.

28. The host cell according to claim 26 wherein the host cell is an antigen presenting cell.

29. The host cell according to claim 28 wherein the host cell is a dendritic cell.

30. A method for treating a host having a tumor expressing CEA or epitope thereof comprising introducing cytotoxic T lymphocytes specific for CEA or epitope thereof to the host and at a periodic interval thereafter introducing to the host an agonist peptide according to claim 1.

31. The method according to claim 30 wherein the peptide is selected from the group consisting of Seq ID Nos: 2, 3, 4, 5 or combination thereof.

32. A method of inhibiting a CEA epitope-expressing carcinoma cells in a patient comprising administering to said patient an effective amount of the peptide according to claim 1.

33. The method according to claim 32 further comprising administration of an immunostimulatory molecule.

34. The method according to claim 33 wherein the immunostimulatory molecule is selected from the group consisting of IL-2, B7.1, B7.2, ICAM-1, LFA-3, CD72, GM-CSF, TNFα, INFγ, IL-12, IL-6 and combinations thereof.

35. The method according to claim 32 further comprising administration of an adjuvant.

36. The method according to claim 32 wherein the carcinoma cell is gastrointestinal, breast, pancreatic, bladder, ovarian, lung, or prostate carcinoma cells.

37. The method according to claim 32 further comprising the administration of a vector comprising the gene encoding CEA.

38. A method of inhibiting or killing CEA epitope-expressing carcinoma cells comprising:

A) generating CEA epitope or agonist peptide-specific cytotoxic T lymphocytes *in vitro* by stimulation of lymphocytes from a source with an effective amount of an agonist peptide according to claim 1 alone or in combination with an immunostimulatory molecule; and

B) adoptively transferring the CEA epitope or agonist peptide-specific cytotoxic T lymphocytes alone or in combination with the agonist peptide into a mammal in an amount sufficient to inhibit or kill the CEA epitope expressing carcinoma cells.

39. A method of inhibiting or killing CEA epitope-expressing carcinoma cells in a mammal comprising:

A) generating CEA epitope or agonist peptide-specific cytotoxic T lymphocytes *in vivo* by administration of an effective amount of a agonist peptide according to claim 1, an effective amount of a vector comprising a nucleic acid sequence encoding CEA or agonist peptide pulsed antigen presenting cells; and

B) at a periodic interval providing the agonist peptide according to claim 1 alone or in combination with an adjuvant;

wherein the CEA epitope or agonist peptide-specific cytotoxic T lymphocytes so generated inhibit or kill CEA epitope-expressing carcinoma cells.

40. A peptide comprising an antagonist of a

native sequence: YLSGANLNL (Seq. ID No: 1) wherein the antagonist varies at at least one amino acid position from SEQ. ID No: 1 and the antagonist inhibits CEA-specific immune responses.

41. A pharmaceutical composition comprising the peptide according to claim 36 and a pharmaceutically acceptable carrier.

42. A method of inhibiting CEA-specific immune responses comprising administration of the peptide according to claim 40 in an amount effective to inhibit the CEA-specific immune responses.

43. The method according to claim 42 wherein cytotoxic T lymphocytes specific for CEA or epitopes thereof are inhibited.

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(57) Abstract

The present invention relates to the preparation and use of peptides that act as agonists and antagonists of human carcinoembryonic antigen (CEA). Agonists of the CEA peptide, CAP1, are disclosed and their utility in enhancing immune responses against CEA demonstrated.

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AGONIST AND ANTAGONIST PEPTIDES OF CARCINOEMBRYONIC ANTIGEN (CEA)

FIELD OF THE INVENTION

The present invention relates to the preparation and use of peptides that can act as agonists and antagonists of human carcinoembryonic antigen (CEA). More specifically, the agonist peptide according to the present invention can be used as an immunogen, either alone, or in prime and boost protocols with other immunogens such as rV-CEA, for a variety of neoplastic conditions. These may include colorectal cancer, lung cancer, pancreatic cancer, and breast cancer. Thus, the present invention also relates to the production and use of vaccines against cancer. Peptide agonists according to the present invention can also be used to facilitate propagation of T cells, for example, from vaccinated patients, for adoptive transfer studies. Peptide antagonists according to the present invention find utility in suppressing autoimmune responses, such as those involving T cells, when such responses occur in vaccinated patients. Thus, the present invention also relates to the production and use of vaccines against autoimmune diseases, especially those mediated by lymphocytes and other antigen presenting cells.

BACKGROUND OF THE INVENTION

A major challenge of modern cancer immunotherapy is the identification of cytotoxic T lymphocyte (CTL) epitopes from defined tumor-associated antigens (TAA) that promote lysis of tumor cells. The majority of antigens on human cancers are not tumor specific and are overexpressed in malignant cells as opposed to cells of normal tissues.

Therefore, immunity to cancer in humans may rest mostly on the development of an effective immune response mainly directed to self-molecules qualitatively common to all cell types.

Human carcinoembryonic antigen (CEA) is a 180 kD glycoprotein expressed on the majority of colon, rectal, stomach and pancreatic tumors (1), some 50% of breast carcinomas (2) and 70% of lung carcinomas (3). CEA is also expressed in fetal gut tissue, and to a lesser extent on normal colon epithelium. The immunogenicity of CEA has been ambiguous, with several studies reporting the presence of anti-CEA antibodies in patients (4-7) while other studies have not (8-10). CEA was first described as a cancer specific fetal antigen in adenocarcinoma of the human digestive tract in 1965 (Gold, P. and Freeman, S.O. (1965) Exp. Med. 121:439-462). Since that time, CEA has been characterized as a cell surface antigen produced in excess in nearly all solid tumors of the human gastrointestinal tract. The gene for the human CEA protein has been cloned. (Oikawa et al (1987) Biochim. Biophys. Res. 142:511-518; European Application No. EP 0346710).

Recently, the first evidence was reported of a human CTL response to CEA (11). This CAP1 peptide showed the highest level of T2 cell binding among the various CEA peptides tested with stimulation of the T cells resulting in the generation of cytotoxic T cell lines. We have identified a 9-mer peptide, designated CAP1 (with the sequence YLSGANLNL) (SEQ. ID NO: 1), on the basis of binding to HLA-A2, and the ability to generate specific CTL from peripheral blood mononuclear cells (PBMC) from carcinoma patients immunized with a recombinant vaccinia

virus expressing CEA (rV-CEA). For example, peripheral blood lymphocytes (PBLs) from 5 patients showed signs of T cell response to CAP1 peptide after immunization with rV-CEA. Two other laboratories have since generated CAP1 specific CTL in vitro employing peptide pulsed dendritic cells as antigen presenting cells (APC) (12). It has also recently been reported (13) that CAP1 specific CTL can be generated from PBMC from carcinoma patients immunized with the avipox recombinant ALVAC-CEA. Several groups have also reported the generation of anti-CEA antibodies and CEA specific proliferative T cell responses following immunization with either an anti-Id to an anti-CEA monoclonal antibody (MAb) (14), recombinant CEA protein (15), or rV-CEA (16).

Several investigators have introduced CTL to tumor associated and viral antigens by in vitro stimulation of PBMC with an immunodominant peptide. Recent work with the gp100 melanoma antigen (17-19), an HIV polymerase peptide (20) and the papilloma virus tumor antigen E6 (21) demonstrated enhanced immunogenicity after modifications to the peptide sequences. In these studies, replacements were at anchor positions and were intended to increase binding to murine or human MHC antigens. This approach was based on a demonstrated correlation between immunogenicity and peptide binding affinity to class I MHC (major histocompatibility complex) molecules for viral antigen epitopes (22).

Previous investigators have also worked with fragments of CEA. Thus, Shively (1989), in a European patent publication (EP No. 0343946 A2) reports a number of CEA fragments that include a unique epitope (as defined by

its reactivity with an antibody). The latter CEA fragment is 177 amino acid residues long and contains the 9-mer sequence of CAP1. However, no shorter CEA fragments that include the CAP1 sequence were described.

In sum, the use of rV-CEA alone as an agent for boosting the CEA-specific immune response of rV-CEA suffers from the drawback of stimulating an immune response to vaccinia virus. However, the novel combination of rV-CEA and CAP1 suggested itself to us as a "second generation protocol" for the treatment of cancer patients.

It is an accepted principle that when an immunogenic peptide is modified in a conserved manner (e.g., a hydrophobic amino acid is substituted with a hydrophobic amino acid) the modified peptide is likely to have similar immunogenic activity based upon the maintenance of the molecule's shape, charge and hydrophobic character.

More specifically, a study by Madden (33) has identified specific amino acid preferences in peptides for MHC-complexing, a precursor step to T cell recognition. Madden as well as other investigators (31) suggest that specific amino acid positions in peptides are available for T cell recognition.

Skipper et al. (40) describes the identification and characterization of a naturally-occurring peptide epitope of tyrosinase, wherein the peptide sequence differs from that which is predicted from the DNA. This modified peptide is recognized by tyrosinase-specific human cytotoxic T-lymphocytes ("CTL") more effectively than the direct translation product and is the only one of the two peptides to be presented by HLA-A2.1 molecules on

the cell surface. The modification is a substitution of an asparagine with an aspartic acid. The authors propose that the asparagine is N-glycosylated in the endoplasmic reticulum during protein synthesis and is deamidated post-translationally.

In the case of CAP1, the primary and secondary anchors at positions 2, 9, and 1 are already occupied by preferred amino acids and so a different approach was taken to improve peptide immunogenicity by attempting to enhance its ability to bind to the TCR. It appeared to us that by altering amino acid residues expected to contact the TCR one could generate an analog of CAP1 with substitutions at non-MHC anchor positions. Such an analog might then represent a T cell enhancer agonist capable of stimulating CTL more efficiently than the native peptide. Previous results supported the concept that some peptide analogs could act as T cell antagonists by inhibiting responses to the antigenic peptide (23-29). Such inhibition was shown to be TCR specific and could not be explained by competition for peptide binding to the MHC protein. Analogously, a peptide enhancer agonist would be an analog that increased the effector function without accompanying increases in MHC binding. We therefore sought to increase CAP1 immunogenicity by analyzing panels of analogs containing single amino acid substitutions to residues we predicted would interact with the T cell receptor (TCR) of CAP1-specific CTL. The present invention relates to the construction of a novel T cell enhancer agonist for the CAP1 peptide, the first such example for a human CTL epitope.

SUMMARY OF THE INVENTION

The present invention relates to the identification of peptides which are single or double amino acid changes from the CAP-1 peptide sequence. The CAP-1 peptide has been identified as a highly immunogenic epitope of the carcinoembryonic antigen (referred to herein as "CEA"), which is capable of stimulating CEA-specific cytolytic T-cell ("CTL") responses. CEA is a cell surface antigen found in abundance on several types of cancer cells. Thus, peptides of CEA capable of stimulating a cytolytic CTL response, such as CAP-1 are potential immunogens for use in cancer immunotherapy.

Some of the peptides of the present invention are agonists of CAP-1 and CEA; that is, they facilitate the interaction between the MHC-complex of the antigen-presenting cell and the T-cell receptor ("TCR") complex of the T-cell. Thus, these peptides can serve as immunogens to treat and/or vaccinate patients with CEA-expressing cancers. Also, these peptides may be used to stimulate T-cells in culture for adoptive transfer of T-cells to cancer patients. Four such peptides have amino acid sequences:

(1) YLSGA<u>D</u>LNL (Agonist CAP1-6D) (SEQ. ID NO: 2);

(2) YLSGA<u>DI</u>NL (Agonist CAP1-6D, 7I) (SEQ. ID NO: 3);

(3) YLSGAN<u>I</u>NL (Agonist CAP1-7I) (SEQ. ID NO: 4); and

(4) YLSGA<u>C</u>LNL (agonist CAP1-6C) (SEQ. ID NO.: 5).

The underlined amino acids identify the amino acids

changes from the CAP-1 peptide sequence. Peptides CAP1-6D and CAP1-6D, 7I are especially preferred peptides according to the present invention and have enhanced activity as compared to CAP-1 activity. Peptides CAP1-7I and CAP1-6C have activity similar to CAP-1.

Other peptides according to the present invention function as antagonists of CEA; that is, they reduce or eliminate CEA-specific T-cell activation and killing which occur through interactions of the MHC-peptide complex and TCR complex.

The present invention encompasses kits comprising an agonist peptide and a vector comprising a gene encoding CEA or a recombinantly produced CEA protein. Moreover, the kit may include an immunostimulatory molecule.

The present invention also encompasses kits comprising an antagonist peptide alone or in combination with an immunosuppressive agent.

Another object of the present invention is a pharmaceutical composition comprising one or more agonist peptides alone or in combination with an immunostimulatory molecule and a pharmaceutically acceptable carrier.

Another object of the present invention is a pharmaceutical composition comprising one or more antagonist peptides alone or in combination with an immunosuppressing agent and a pharmaceutically acceptable carrier.

The present aspect of the present invention is a nucleic acid sequence encoding at least one agonist peptide or encoding at least one antagonist peptide.

Another aspect of the invention is a vector

comprising a nucleic acid sequence encoding at least one agonist peptide or a nucleic acid sequence encoding at least one antagonist peptide and host cells comprising such vectors.

Another aspect of the present invention relates to the use of these peptides in cancer immunotherapy. The agonist peptides are useful in stimulating a cytolytic immune response to CEA, resulting tumor reduction and/or prevention. Accordingly, the present invention also relates to a method of treating cancer patients with the peptides as well as a cancer vaccine. The antagonist peptides are useful in methods of controlling autoimmune response to CEA or CAP-1.

Yet another aspect of the present invention is an agonist-pulsed antigen presenting cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-1D: Effect of single amino acid substitutions in CEA CAP1 peptide on lysis by CEA CTL T-Vac8 C1R-A2 cells were labeled with ^{111}In and incubated for 1 hour in round bottom wells (2,000/well) with each substituted peptide at 1 (solid), 0.1 (open) and 0.01 (hatched) μg/ml. T-Vac8 CTL were added at E:T=1.45:1 and isotope release was measured after 4 hours. Spontaneous release was determined for each peptide at 1 μg/ml. All assays were performed in triplicate. Figures 1A-1D depict substitutions at positions p5 through p8, respectively. Amino acids are designated by the single letter code; the amino acid encoding the native CAP1 sequence is indicated in each figure and along the right-hand margin.

Figure 2A and 2B: CAP1 and analogs show different sensitivity to CEA CTL T-Vac8 cytotoxicity Figure 2A T2

and Figure 2B C1R-A2 target cells were labeled with ^{51}Cr and incubated in round-bottomed 96 well plates (10,000/well) with CAP1 (●) or substituted peptides CAP1-6D (□) or CAP1-7I (◊) at the indicated concentrations. After 1 hour, T-Vac8 CTL were added at E:T=2.5:1 and isotope release was determined after 4 hours. All assays were done in triplicate. NCA571 (Δ) is a 9-mer peptide obtained after optimal alignment of CEA with the related gene NCA (11).

Figure 3: Effect of single amino acid substitutions in CAP1 peptide on binding to and stability of HLA-A2 complexes T2 cells were collected in serum free medium then incubated overnight (10^6 well) with peptides CAP1(●), CAP1-6D (□), or CAP1-7I (◊) at the indicated concentrations. Cells were collected and assayed for cell surface expression of functional HLA-A2 molecules by staining with conformation sensitive MAb BB7.2, HLA specific antibody W6/32 (not shown) and isotype control Ab MOPC-195 (not shown). Mean fluorescent intensity was determined on a live, gated cell population.
Figure insert: Cells were incubated with peptide at 100 μg/ml overnight, then washed free of unbound peptide and incubated at 37°C. At the indicated times, cells were stained for the presence of cell surface peptide-HLA-A2 complexes. The error bars indicate SEM for two experiments.

Figure 4A and 4B: CTL generated from apparently healthy individuals with CAP1-6D peptide recognize CAP1 and CAP1-6D CTL lines (designated T-N1 and T-N2) were generated with CAP1-6D and were assayed for peptide specificity. T-

N1 was assayed after 5 cycles of stimulation at an effector to target ratio of 20:1 (Figure 4A). T-N2 was assayed after 10 cycles at an effector to target ratio of 15:1 (Figure 4B). ^{51}Cr-labeled C1R-A2 targets (5,000/well) were incubated with the indicated amount of CAP1 (•) or CAP1-6D (□) peptide. After 4 hours the amount of isotope release was determined in a gamma counter. Values were determined from triplicate cultures.

Figure 5A and 5B: CAP1-6D, but not CAP1 generated T cell lines from apparently healthy donors recognize tumor cells expressing endogenous CEA CAP1-6D generated T-N2 CTL (Figure 5A) and T cells generated with native CAP1 (Figure 5B), were assayed after 9 cycles of in vitro stimulation against tumor targets SW480 and SW1463 (CEA^+, $HLA\text{-}A2^+$, ● and ▲ respectively), SKmel24 (CEA^-, $-A2^+$, □) and K562 (◊). Tumor cells were cultured for 72 hours in the presence of γ-IFN to up regulate HLA. Cells were trypsinized and labeled with ^{51}Cr and incubated (5,000 cells/well) with T-N2 CTL at increasing effector to target ratios. Cultures were incubated for 4 hours and the amount of isotope release determined in a gamma counter. Values were determined from triplicate cultures.

Figure 6: MHC-class 1 A2.1 restriction of CTL line (T-N2) derived from CAP1-6D agonist CTL line T-N2 was used as an effector for the human colon carcinoma SW837 target cell. SW837 is CEA positive and HLA-A2.1 negative. SW837 were infected at an MOI of 10:1 with either a recombinant vaccinia containing the A2.1 transgene (■) or wild type vaccinia (Δ).

Figure 7A and 7B: CTL generated with CAP1-6D lyse CEA positive, HLA-A2 positive tumors: Effect of IFN

upregulation The T-N1 CTL generated with CAP1-6D were assayed against various tumor cell lines: SW480 (CEA^+ and $HLA\text{-}A2^+$, ●), SW1116 (CEA^+ but $-A2^-$, □) and CaOV3 (CEA^- but $-A2^+$, ◊). Tumor cells were cultured 72 hours in the absence (Figure 7A) or presence (Figure 7B) of γ-IFN, trypsinized and labeled with ^{51}Cr then incubated (5,000 cells/well) with T-N1 CTL at increasing effector to target ratios. Cultures were incubated for 4 hours and the amount of isotope release determined in a gamma counter. Values were determined from triplicate cultures.

DETAILED DESCRIPTION OF THE INVENTION

The invention is an peptide agonist of the native CEA epitope, CAP-1 (SEQ. ID NO: 1), as well as antagonists of SEQ. ID NO: 1. The agonist is characterized by its ability to elicit antigen specific cytotoxic T lymphocytes which inhibit the growth or kill carcinoma cells expressing CEA or CEA epitopes. An antagonist of the present invention serve to inhibit or prevent CEA specific immune responses. Such peptides may be used to shut off any unwanted immune responses to CAP-1 or CEA. One example for such use of an antagonist is to control any possible autoimmune response that may occur during cancer immunotherapy, where the therapy has killed off tumor cells and begins to attack normal cells expressing CEA. In accordance with the present invention an antagonist would advantageously prevent extensive damage to normal tissue.

The peptide agonists of the present invention comprise about 8-13 amino acids, preferably 9-10 amino acids. In a preferred embodiment, the agonist peptide of the present invention comprises at least one amino acid

substitution at a non-anchor position. In one embodiment, the agonist comprises a sequence with a substitution at position 6 compared to the native CAP-1 (SEQ. ID NO: 1). In another embodiment the agonist comprises a sequence with an amino acid substitution at position 7 compared to the native CAP-1 (SEQ. ID NO: 1). In yet another embodiment, the agonist comprises a sequence with an amino acid substitution at position 6 and at position 7 compared to the native CAP-1. The substituted amino acid serves to enhance the interaction of the TCR complex on the cytotoxic T lymphocytes with the peptide- MHC antigen ligand complex. Such enhanced interaction results in greater effector function by the cytotoxic T lymphocytes.

An example of a substitution includes Asp and Cys at position 6 or an Ile at position 7.

In one embodiment, the peptide agonist comprises the following amino acid sequence:

Amino Acid Position	1	2	3	4	5	6	7	8	9	
Native CAP-1 Peptide	Y	L	S	G	A	N	L	N	L	(SEQ. ID NO: 1)
Agonist	Y	L	S	G	A	D	L	N	L	(SEQ. ID NO: 2)
Agonist	Y	L	S	G	A	D	I	N	L	(SEQ. ID NO: 3)
Agonist	Y	L	S	G	A	N	I	N	L	(SEQ. ID NO: 4)
Agonist	Y	L	S	G	A	C	L	N	L	(SEQ. ID NO: 5)

The agonist peptide of the present invention may be obtained by recombinant DNA technology or by chemical peptide synthesis.

The agonist peptide may be formulated into a pharmaceutical composition in combination with a pharmaceutically acceptable carrier for use as an

immunogen in a mammal, preferably a human. The composition may further comprise one or more other constituents to enhance the immune response which include but are not limited to immunostimulatory molecules such as interleukin 2, interleukin 6, interleukin 12, interferon gamma, tumor necrosis factor alpha, GM-CSF, B7.1, B7.2, ICAM-1, LFA-3, CD72, and cyclophosphamide.

The agonist peptide is administered to a mammal in an amount effective in generating a CEA specific immune response, preferably a cellular immune response. The efficacy of the mutant *ras* peptide as an immunogen may be determined by *in vivo* or *in vitro* parameters as are known in the art. These parameters include but are not limited to antigen specific cytotoxicity assays, regression of tumors expressing CEA or CEA epitopes, inhibition of cancer cells expressing CEA or CEA epitopes, production of cytokines and the like.

At least one or more agonist peptides may be administered in a dose of about 0.05 mg to about 10 mg per vaccination of the mammal, preferably about 0.1 mg to about 5 mg per vaccination. Several doses may be provided over a period of weeks as indicated. In one embodiment a dose is provided every month for 3 months. The agonist peptide may be administered alone or in combination with adjuvants, incorporated into liposomes (U.S. Patent Nos. 5,643,599; 5,464,630; 5,059,421; 4,885,172), with cytokines, biological response modifiers, or other reagents in the art that are known to enhance immune response. Adjuvants include but are not limited to RIBI Detox™, QS21, alum and incomplete Freund's adjuvant. In one embodiment, the mutant *ras* peptide is administered in

combination with Detox™ (RIBI Immunochem Research, Hamilton, MT). RIBI Detox™ contains as active ingredients the cell wall skeleton from *Mycobacterium phlei* and monophosphoryl lipid A from *Salmonella minnesota* R595 prepared as an oil-in-water emulsion with squalene and tween 80.

The agonist peptides may also be conjugated to helper peptides or to large carrier molecules to enhance the immunogenicity of the peptide. These molecules include but are not limited to influenza peptide, tetanus toxoid, tetanus toxoid CD4 epitope, Pseudomonas exotoxin A, poly-L-lysine, a lipid tail, endoplasmic reticulum (ER) signal sequence and the like.

The peptides of the present invention may also be conjugated to an immunoglobulin molecule using art accepted methods. The immunoglobulin molecule may be specific for a surface receptor present on tumor cells but absent or in very low amounts on normal cells. The immunoglobulin may also be specific for a specific tissue. Such a peptide-immunoglobulin conjugate allows for targeting of the peptide to a specific tissue and/or cell.

Another effective form of the agonist peptide for generating an peptide specific immune response in a mammal is an agonist peptide-pulsed antigen presenting cell. The antigen presenting cells include but is not limited to dendritic cells, B lymphocytes, monocytes, macrophages and the like. In a preferred embodiment, the agonist peptide-pulsed antigen presenting cell is a dendritic cell.

The invention also provides a method of generating CEA and agonist peptide specific cytotoxic T

lymphocytes *in vivo* or *in vitro* by stimulation of lymphocytes from a source with an effective amount of a agonist alone or in combination with a immunostimulatory molecule and/or adjuvant or in a liposome formulation. The sources of lymphocytes include but are not limited to peripheral blood, tumor tissues, lymph nodes and effusions such as pleural fluid or ascites fluid and the like.

The CEA and agonist peptide specific cytotoxic T lymphocytes of the present invention are immunoreactive with CEA agonist or peptide. The cytotoxic T lymphocytes inhibit the occurrence of tumor cells and cancer and inhibit the growth or kill expressing tumor cells expressing CEA or eptiopes thereof or agonist expressing tumor cells. The cytotoxic T lymphocytes, in addition to being antigen specific, are MHC class I restricted. In one embodiment the cytotoxic T lymphocytes are MHC class I HLA-A2 restricted. The cytotoxic T lymphocytes have a $CD8^+$ phenotype.

Selected patients bearing carcinoma cells expressing CEA or CEA epitopes are vaccinated subcutaneously up to three times at monthly intervals with DETOX™ adjuvant admixed with the appropriate peptide agonist may also be vaccinated carcinoma patients with autologous peripheral blood mononuclear cells pre-pulsed ex vivo with a peptide agonist alone or in combination with a peptide agonist. Anti-CEA T cell responses are evaluated as measured by proliferation assays.

Vaccination with CEA agonist peptides of the present invention induces highly specific and systemic anti-CEA cellular immune responses. Moreover, the development of such MHC class I-restricted agonist peptides has important

implications for both active (i.e., vaccination) and passive (i.e., ex vivo expansion for cellular adoptive transfer) immunotherapies, which may be used for the induction and propagation of specific $CD8^+$ CTL responses in cancer patients.

Patients with solid tumors expressing CEA or epitopes thereof, including but not limited to colon cancer, lung cancer, pancreas cancer, endometrial cancer, breast cancer, thyroid cancer, melanoma, oral cancer, laryngeal cancer, seminoma, hepatocellular cancer, bile duct cancer, acute myeloblastic leukemia, basal cell carcinoma, squamous cell carcinoma, prostate cancer and the like benefit from immunization with the agonist peptides. Patients amenable to treatment using the agonist peptides of the present invention are those patients having tumors with CEA or CEA epitopes.

Peptides may be chemically synthesized under GMP conditions and purified by HPLC to >95% purity and lyophilized. Pharmaceutical compositions are formulated by reconstituting the peptide with a pharmaceutically acceptable carrier such as sodium chloride. In one example, each milliliter of solution contains 1500 μg of a agonist peptide plus 9.0 mg sodium chloride.

When the agonist peptide is administered with an adjuvant it is desirable to mix the peptide with the adjuvant shortly before administration to a patient.

The agonist peptide may be administered to a patient by various routes including but not limited to subcutaneous, intramuscular, intradermal, intraperitoneal, intravenous and the like. In one embodiment the agonist peptide is administered subcutaneously. The peptide may

be administered at one or more sites to a patient. In one embodiment, the peptide, alone or in combination with an adjuvant, is administered into three sites subcutaneously, over the deltoids, the thighs and the abdomen.

In another method of generating an immune response, agonist peptide-pulsed antigen presenting cells are administered to the patient in an amount effective to generate an antigen specific immune response. The antigen presenting cells include but are not limited to dendritic cells, B lymphocytes, monocytes, macrophages and the like. In one embodiment, dendritic cells are isolated from a patient by methods described in Romani, N. et al (1994). The isolated dendritic cells are cultured *in vitro* with an agonist peptide for a period of about 0.5 to about 3 hours and washed to remove non-bound peptide. The agonist peptide-pulsed dendritic cells are transferred back into the patient at a concentration of about 10^6 to about 10^9 dendritic cells. Such a concentration is effective in generating an immune response in the patient including the generation of agonist peptide specific cytotoxic T lymphocytes which are able to inhibit the growth or kill tumor cells.

The criteria for determining an anti-tumor response in the immunized patient is as follows:

1. Complete Remission (CR): Complete disappearance of all evidence of tumor and return of abnormal tests to normal levels for a minimum of 4 weeks.

2. Partial Response (PR): Decrease by at least 50% in the sum of the products of the perpendicular diameters of all measured lesions in the absence of progression of any lesion nor the appearance of any new

lesions for at least 4 weeks.

3. Stable Disease (SD): Change in measurable disease too small to meet the requirements for partial response or progression and the appearance of no new lesions for a period of at least 12 weeks. There may be no worsening of symptoms.

4. Progressive Disease (PD) or Relapse: Any one of the criteria below must be met to be considered progressive disease:

Development of any new area of malignant disease (measurable or palpable),

Increase (>25%) in any pretreatment area of measurable malignant disease.

The immunological response to immunization with the agonist peptides are assessed by in-vitro T cell proliferation assay and/or by in-vitro T cell cytotoxic assay before and after vaccination.

The present invention includes *in vitro* immunization for T cell proliferation and generation of cytotoxic T cell lines to the tumor specific agonist peptide. *In vitro* cultivation of peptide specific T cells from peripheral blood mononuclear cells (PBMC), lymph node tissue (LNT), or tumor infiltrating lymphocytes (TIL) with agonist peptide and IL-2 generates CEA and agonist peptide specific T cells. These T cells are tested for cytotoxicity against agonist peptide primed APC (autologous EBV transformed B cells or autologous tumor cells) has described herein. Generated T cell clones are characterized phenotypically by flow cytometry for express of CD3, CD4, and CD8. Agonist peptide specific cytotoxic lymphocytes may be adoptively transferred to a patient in

order to inhibit or kill CEA or CEA epitopes expressing tumor cells. Patients may then be reimmunized with agonist peptide preferably in adjuvant.

Generally, between about 1 x 10^5 and 2 x 10^{11} cytotoxic T cells per infusion are administered in, for example, one to three infusions of about 200 to about 250 ml each over a period of 30 to 60 minutes. After completion of the infusions, the patient may be treated with a biological response modifier such as interleukin 2 (IL-2). In the case of IL-2, recombinant IL-2 is administered intravenously in a dose of 720,000 IU per kilogram of body weight every eight hours. After adoptive transfer of the antigen specific cytotoxic T cells into the patient, the patient may be additionally treated with the agonist peptide used to prime the cytotoxic T cells, to further expand the T cell number *in vivo*.

The invention encompasses a DNA sequence and variants thereof which encode an agonist peptide.

In one embodiment the DNA sequence encoding the agonist peptide is a variant of the DNA sequence comprising:

TAC CTT TCG GGA GCG AAC
Tyr Leu Ser Gly Ala Asn

CTC AAC CTC (SEQ. ID No: 6)
Leu Asn Leu (SEQ. ID No: 1).

One variant of SEQ. ID No: 6 includes but is not limited to a codon ATC (Ile) in place of the codon, CTC (Leu at position 7). Another variant of SEQ. ID No: 6 includes but is not limited to a codon, TGT (Cys) in place of the codon, AAC (Asn at position 6).

In another embodiment, the DNA sequence encoding the agonist peptide comprises:

TAC CTT TCG GGA GCG GAC
Tyr Leu Ser Gly Ala Asp

CTC AAC CTC (SEQ. ID No: 7)
Leu Asn Leu (SEQ. ID No: 2)
and variants thereof.

In yet another embodiment, the DNA sequence encoding the agonist peptide comprises:

TAC CTT TCG GGA GCG GAC
Tyr Leu Ser Gly Ala Asp

ATC AAC CTC (SEQ. ID No: 8)
Ile Asn Leu (SEQ. ID No: 3)
or variants thereof.

Included in the ambit of the invention are conservative substitutions based on codon degeneracy provided the modification results in a functionally equivalent agonist peptide or a peptide with enhanced immunogenicity.

The invention further provides vectors and plasmids comprising a DNA sequence encoding an agonist peptide. The vectors include but are not limited to E. coli plasmid, a Listeria vector and recombinant viral vector. Recombinant viral vectors including but not limited to orthopox virus, avipox virus, capripox virus, suipox virus, vaccinia, baculovirus, human adenovirus, SV40, bovine papilloma virus, and the like comprising the DNA sequence encoding an agonist peptide.

Recombinant agonist peptide can be obtained

using a baculovirus expression system in accordance with the method of Bei et al J. Clin. Lab. Anal. 9:261-268 (1995). Recombinant viral vectors can be constructed by methods known in the art such as U.S. Patent No. 5,093,258; WO96/10419 Cepko et al Cell 37:1053-1062 (1984); Morin et al Proc. Natl. Acad. Sci USA 84:4626-4630 (1987); Lowe et al Proc. Natl. Acad. Sci USA 84:3896-3900 (1987); Panicali & Paoletti, Proc. Natl. Acad. Sci USA 79:4927-4931 (1982); Mackett et al, Proc. Natl. Acad. Sci USA 79:7415-7419 (1982); WO 91/19803; Perkus et al Science 229:981-984 (1985); Kaufman et al Int. J. Cancer 48:900-907 (1991); Moss Science 252:1662 (1991); Smith and Moss BioTechniques Nov/Dec, p. 306-312 (1984); U.S. Patent No. 4,738,846; Sutter and Moss Proc. Natl. Acad. Sci USA 89:10847-10851 (1992); Sutter et al Virology (1994); and Baxby and Paoletti Vaccine 10:8-9 (1992).

Host cells which may express the DNA encoding the agonist peptide carried by vectors or plasmids are prokaryotic and eukoryotic host cells and include but are not limited to E. coli, Listeria, Bacillus species, COS cells, Vero cells, chick embryo, fibroblasts, tumor cells, antigen presenting cells and the like. When the host cell is an antigen presenting cell, the host cell is an antigen presenting cell, the host cell should additionally express an MHC class I molecule.

We recently reported (11) evidence of CTL responses to CEA in patients immunized with rV-CEA. The 9-mer peptide CAP1 was employed to expand CTL in vitro because of: (a) its strong binding to HLA-A2, and (b) its non-identity to other members of the CEA gene family expressed on normal tissues. CTLs were generated from

post-immunization PBMC of patients while preimmunization blood of the same patients failed to proliferate. In addition, CAP1 pulsed dendritic cells stimulated in vitro growth of -A2 restricted CTL from peripheral blood of unimmunized cancer patients (12). Finally when CTL were generated in vitro by stimulation with dendritic cells encoding full-length CEA mRNA, cytotoxicity against CAP1 was higher than activity against six other -A2 binding CEA peptides (S. Nair and E. Gilboa, personal communication or unpublished observation). Such results encourage the notion that CAP1 is an immunodominant epitope of the CEA molecule.

The present invention is intended to improve the immunogenicity of the CAP1 peptide by introducing amino acid substitutions at non-anchor positions to form the agonist peptides of the present invention. When using T-Vac8 CTL as an effector, the analog CAP1-6D sensitized target cells for lysis far better than CAP1 itself. Further studies showed that cytolytic activity of a second -A2 restricted, CAP1 specifIc CTL, T-Vac24, was as good or greater with CAP1-6D than with CAP1. These demonstrations of enhanced reactivity could not be explained by improved presentation by class I MHC. Finally, CAP1-6D could be used to stimulate CTL in vitro from PBMC of both carcinoma patients and normal donors. Prior to the present invention, attempts to stimulate anti-CAP1 CTL from normal donors using this same methodology have been unsuccessful. The present invention relates to stimulation of normal donors with CAP1-6D as opposed to native CAP1 where stimulation with the native sequence failed to produce specific cytotoxic activity. In contrast, stimulation with

CAP1-6D produced several CTL with specific anti-CAP1 peptide reactivity as well as anti-tumor reactivity. Thus, the analog peptide CAP1-6D is capable of selecting a population of CAP1 specific human CTL more efficiently than native CAP1. Such an agonist might find applications in the design of T cell directed vaccines against CEA-expressing carcinoma.

The present invention also relates to the more efficient generation and expansion of tumor specific T cells for adoptive immunotherapy. In recent years, much progress has been achieved in characterizing the tumor associated antigen peptides that can be presented to CTL by class I HLA antigens. In instances where mutations generate neo-antigens such as point mutated ras (35, 36), p53 (37, 38) or ß-catenin (39) vaccination strategies target the novel sequence under the assumption that the immune system is not "tolerant" to an antigen it has never seen. More recently it has been proposed that neo-antigens may also arise through post-translational deamidations (29, 40). However, in many instances the intended targets of tumor therapy are not neoantigens but rather normal oncofetal or differentiation antigens that are overexpressed or ectopically expressed by malignant cells. Such is the case for CEA (41). In such situations, models invoking "tolerance" predict that the immune system has encountered these antigens and is less able to respond to them. This classical picture has been challenged in recent years by numerous reports of immunity elicited to overexpressed differentiation antigens, oncogenes, and tumor suppressor genes (37, 38, 42-44). Nonetheless, it is often experimentally difficult to generate and expand T

cells with desired anti-tumor activity and it is therefore desirable to devise new strategies for generating CTL.

Some class II binding-peptides have been described in which substitutions enhance responses of murine and human Th clones without increasing the binding to class II antigens (29, 45-47). Among human class I peptides, however, the only substitutions described for the generation of CTL were those that increase binding to HLA (17-20). The substitutions in those studies were directed to residues at the primary or secondary anchor positions that define the binding motifs to class I MHC antigens. Even substitutions directed to a non-anchor position (19) achieved their enhancing effect by increasing binding to HLA-A2. The analog CAP1-6D in the present report represents what appears to be a different class of substituted CTL peptides, agonists that enhance recognition of the peptide-MHC ligand by the T cell receptor and produce greater effector function without increases in binding. To our knowledge this is the first such enhancer agonist peptide described for a human CTL.

The increased lytic susceptibility of targets in the presence of CAP1-6D is unlikely to be due to better antigen presentation. Binding experiments show that HLA-A2 presents the native CAP1, and the analogs CAP1-6D and CAP1-71 approximately equally. Another possibility is that CAP1-6D shows increased activity because it is presented by more than one allele and T-Vac8 is promiscuous towards peptide-MHC complexes. However, T-Vac8, T-Vac24, and CTL derived from nonimmunized patients showed better lysis with CAP1-6D. Since HLA-A2 is the only class I MHC on the targets employed, the improved

lysis cannot be accounted for by recruitment of another class I MHC.

Since anti-CAP1 CTL from multiple donors demonstrate agonist cross reactivity it is possible that CAP1-6D could be used to stimulate growth of CTL from numerous -A2 individuals. We are encouraged by the quite distinct differences between T-Vac8 and T-Vac24 in magnitude of response to the agonist; this implies that each effector utilizes different TCR gene segments and that nonetheless they can recognize both the native sequence and the CAP1-6D substitution. The ability of CAP1-6D to act as an agonist with T cells expressing different T cell receptors clearly magnifies its therapeutic potential. Thus, the present invention also relates to stimulation with the agonist and subsequent generation of T cells that recognize the normal sequence in non-immunized individuals. Such individuals have presumably never encountered the modified sequence and since the agonist is more efficient at triggering a T cell response, such agonists might be capable of selecting CTL more readily than immunogens based on the native sequence.

For peptide-derived CTL to be useful therapeutic reagents it is essential to demonstrate that they can lyse tumor cells that express endogenous antigen (48, 49). Previously (11), we had shown that tumor cells process CEA and present antigens recognized by CTL generated by stimulation with CAP1. In accordance with the present invention, CTL grown from the normal donors by stimulation with CAP1-6D are also capable of recognizing allogeneic CEA-positive, HLA-A2 positive tumor cells. These T cells fail to recognize -A2 negative tumor cells or -A2 positive

cells that lack CEA expression.

We have also shown that CTL selected with the CAP1-6D agonist can be maintained subsequently by stimulation with the native CAP1 sequence. This is an important finding since CTL in patients, whether established in vivo through active immunization, or transferred adoptively after ex vivo expansion, will likely only encounter the native sequence. This allows the CTLs to be maintained over an extended duration in vivo.

One of the original reasons for selecting and testing CAP1 was its non-identity with other reported sequences in the human genome. It was therefore predicted that any immune responses attained would be unlikely to damage normal tissues bearing other antigens. For this reason a similar search of protein databases was undertaken for the peptides CAP1-6D and CAP1-7I and revealed that they are not reported as human sequences elsewhere in the Genebank (Genetics Computer Group, Madison, WI). However, two similar sequences, YLNVQDLNL (SEQ. ID No: 9) and YLHDPEFNL (SEQ. ID No: 10), are reported for antigens from African swine fever virus and measles virus, respectively. These sequences fit the consensus motif for HLA-A2 and therefore allow infected individuals to express cross-reacting antigens to CAP1. One interesting possibility is that the presence of anti-CAP1 CTL in some patients represents an example of epitope mimicry (50).

Two recent reports suggest that modified asparagine residues might enhance the immunogenicity of class I MHC peptides. Skipper et al. (40) used CTL generated in mixed lymphocyte tumor cell cultures to

identify antigens in extracts of melanoma cells. One antigenic peptide was identical at 8 of 9 positions to a sequence from tyrosinase, with an asparagine to aspartic acid replacement at position 3. When tested using synthetic peptides, the CTL were more active against the aspartic acid peptide than against the peptide containing the genetically predicted asparagine. These authors speculate that post-translational deamidations can generate antigenic peptides from normal differentiation antigens. Recently, Chen et al. (51) reported generating murine CTL to a stabilized succinimide derivative of an asparagine-containing antigenic peptide. Although these CTL could kill targets pulsed with the natural asparagine peptide, they did so with less sensitivity. They raise the possibility that deamidation of proteins in vivo and in vitro can produce transient succinimide intermediates that represent altered self-ligands capable of eliciting an immune response. At the other extreme, Kersh and Allen (52) replaced a TCR contact asparagine with aspartic acid in a hemoglobin peptide and abolished responsiveness to a murine Th clone. Presently we cannot exclude the possibility that the enhanced reactivity of CAP1-6D is due to deamidation of the native sequence which in turn primes the response that we detect with CAP1. However, our repeated inability to raise anti-CAP1 CTL from pre-immunized PBMC of the same patients from whom we generated post-immunization CTL, argues against this. Also, putative deamidations could not account for the recognition of other analogs such as CAP1-6C or CAP1-7I by T-Vac8 CTL. Instead it seems more reasonable that T cell receptors from both T-Vac8 and T-Vac24, as well as the new

lines described here, can recognize some deviation from the native CAP1 sequence.

In summary, synthesis of analogs of an immunodominant CEA peptide with amino acid substitutions at positions predicted to potentially interact with the T cell receptor allowed us to identify an enhancer agonist. This agonist was recognized by two different CEA CTL and increases the activity of one of them by 2-3 orders of magnitude. The agonist was also able to stimulate growth of CTL from peripheral blood of non-immunized normal donors with far greater facility than the native peptide sequence. Most important, the CTL generated using the enhancer agonist was able to recognize and lyse targets presenting the native sequence, including tumor cell lines expressing endogenous CEA. In accordance with the present invention, characterization of this enhancer agonist peptide facilitates more aggressive anti-tumor immunotherapies when employed as an immunogen in vivo, or for the ex vivo expansion of autologous anti-tumor CTL. The synthetic approach employed according to the present invention is also useful in improving immunogenicity of other peptide CTL epitopes.

MATERIALS AND METHODS

PEPTIDES

A panel of single amino acid substitutions to positions p5 through p8 of the CEA peptide CAP1 were made by f-moc chemistry using pin technology (Chiron Mimotopes, Victoria, Australia). CAP1 (YLSGANLNL) and CAP1-6D (YLSGADLNL), greater than 96% pure, were also made by Multiple Peptide Systems (San Diego, CA). Additional peptides CAP1-7I and NCA571 were synthesized on an Applied

Biosystems 432A synthesizer and were greater than 90% pure by C18 reverse-phase HPLC.

CELL LINES

T-Vac8 (53) and T-Vac24 (11) are human CTL specific for the CEA peptide CAP1. These cell lines were generated by in vitro stimulation of PBMC using CAP1 and IL-2, according to previously published methods (11). Briefly, post-immunization PBMC were from HLA-A2+ individuals with advanced carcinoma that had been administered rV-CEA in a Phase I trial. PBMC were isolated on gradients of lymphocyte separation medium (Organon Teknika, Durham, NC) and $2x10^5$ cells were placed in wells of sterile 96 well culture plates (Coming Costar, Cambridge, MA) along with 50 µg/ml peptide. After 5 days incubation at 37°C in a humidified atmosphere containing 5% CO_2, supernatants were removed and replaced with medium containing 10 U/ml human IL-2 (a gift of the Surgery Branch, NCI). Cultures were fed with IL-2 every 3 days for 11 days and then restimulated with irradiated (4000 rad) autologous PBMC ($5x10^5$) and peptide. Fresh IL-2 was provided every third day and subsequent restimulations were done every 2 weeks. CTL are maintained in complete RPMI (GIBCO/BRL, Grand Island, NY) medium with glutamine (GIBCO/BRL), penicillin, streptomycin and 10% pooled human AB serum (Gemini Bioproducts, Inc., Calabasas, CA).

Cell line C1R-A2 (provided by Dr. W. Biddison, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD) is maintained in complete RPMI with 10% fetal bovine serum (FBS, Biofluids Inc., Rockville, MD), glutamine, non essential

amino acids and pyruvate (Biofluids) and 1 mg/ml G418. Cell line 174.CEM-T2 (provided by Dr. P. Creswell, Yale University School of Medicine, New Haven, CT) is defective in endogenous peptide processing and is maintained in Iscove's (GIBCO/BRL) with 10% FBS. Both C1R-A2 and T2 lines present exogenous peptides with HLA-A2.

CEA positive tumor cell lines SW480, SW1463, SW1116 and SW 837 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and passaged weekly in respective culture medium described in the ATCC catalog. The CEA negative melanoma line SKmel24 (provided by Dr. S. Rosenberg, National Cancer Institute, National Institutes of Health, Bethesda, MD) was passaged weekly in RPMI 1640, 10% FBS and 10 μg/ml gentamicin (Life Technologies). The CEA negative ovarian tumor CaOV3 was provided by Dr. R. Freedman (MD Anderson Cancer Center, Houston TX) and was cultured in RPMI with 15% FBS, glutamine, 12 μg/ml insulin (Sigma, St. Louis, MO), 10 μg/ml hydrocortisone (Biofluids) and 10 μg/ml gentamicin. All tumor lines were trypsinized with Trypsin/Versene (Biofluids) for 5-10 minutes prior to labeling with isotope for CTL assays. The highly sensitive natural killer (NK) target K562 was obtained from ATCC and passaged weekly with RPMI 1640, 10% FBS.

GENERATION OF CTL

T cell lines T-N1 and T-N2 were generated from PBMC of two normal HLA-A2 positive donors by in vitro stimulation with peptide as follows. For the first stimulation cycle, T cells were positively selected by panning on CD3+ MicroCellector flasks (Applied Immune

Sciences, Santa Clara, CA). CD3+ cells ($3x10^6$) were cultured with 10^6 174.CEM-T2 cells that were previously infected with vaccinia virus expressing human B7 at a multiplicity of infection of 10, pulsed with 50 μg/ml CAP1 or CAP1-6D peptide and 2 μg/ml human ß2 microglobulin (Intergen, Purchase, NY), and irradiated (10,000 rad). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2, in T25 flasks in RPMI with 10% human serum, 2 mM glutamine, and 10 μg/ml gentamicin in a total volume of 10 ml with $2x10^7$ irradiated (2500 rads) autologous PBMC as feeder cells. After 24 hours in culture 10 U/ml hulL-2 and 0.1 ng/ml rIL-12 (R & D Systems, Minneapolis, MN) were added. After 9 days in culture, cells were restimulated using irradiated (10,000 rads) autologous EBV-B cells preincubated with 25 μg/ml peptide at a ratio of 2.5:1 stimulator cells to T cells, and IL-2 and IL-12 were again added 24 hours later. Peptide concentration was halved with each subsequent stimulation cycle until a final concentration of 3.12 μg/ml was achieved.

In addition, CTL were generated from post-immunization PBMC of cancer patient Vac8 by stimulation with CAP1-6D according to already published procedures (11).

CTL ASSAY

Target cells were labeled with ^{51}Cr or ^{111}In, then incubated at 2,000-10,000 per well with or without peptides in round bottom microtiter plates (Corning Costar). One hour later, T cells were added. Supernatants were harvested (Skatron, Inc., Sterling VA)

after 4 hour and isotope release was measured. All assays were performed in triplicate and percent specific release was calculated according to:

$$\frac{\text{(observed release-spontaneous release)}}{\text{(maximum release-spontaneous release)}} \times 100$$

where spontaneous release is obtained by omitting the T cells, and maximum release is obtained by adding 1% Triton X100.

BINDING ASSAY

Binding of peptides to HLA-A2 was evaluated by incubation with processing defective 174.CEM-T2 cells and measuring the stability of cell surface peptide-A2 complexes (30). Briefly, cells were harvested and washed with serum-free RPMI then incubated overnight at 1-2x10^6 cells/well with various concentrations of peptides. The next day, cells were collected, washed in PBS with Ca^{2+}, Mg^{2+} and 5% FBS, then divided into aliquots for single color flow cytometric analysis. Cells were incubated 1 hour on ice without antibody, with anti-A2 antibody A2,69 (One Lambda, Inc., Canoga Park, CA) or with isotype-matched control antibody UPC-10 (Organon Teknika) then washed and stained 1 hour with fluorescein-isothiocyanate (FITC) goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL). Cell surface staining was measured in a Becton Dickinson flow cytometer (Mountain View, CA) and the mean fluorescence intensity (MFI) for 10,000 live cells was plotted against peptide concentration.

TCR CHAIN USAGE

T-N1 CTL were cultured as described for 5 cycles of antigenic stimulation using the CAP1-6D analog. The line was then split and duplicate cultures were maintained either with CAP1 or CAP1-6D for 5 additional stimulation cycles. Ficoll-purified T cells ($5x10^5$) were stained with a panel of 19 anti-Vß and 2 anti-Vα murine monoclonal antibodies to human αß T cell receptor variable regions. Cells were incubated with 10 μg/ml of purified antibodies for 30 minutes at 4°C. The unlabeled monoclonals used were: Vß3.1 clone 8F10, Vß5(a) clone 1C1, Vß5(b) clone W112, Vß5(c) clone LC4, Vß6.7 clone OT145, Vß8(a) clone 16G8, Vß12 clone S511, Vß13 clone BAM13, Vα2 clone F1 and Vα12.1 clone 6D6 (T Cell Diagnostics, Woburn, MA) and Vß18 (Immunotech, Westbrook, ME). Cells were stained with 10 μg/ml of FITC-labeled goat anti-mouse IgG antibody (Southern Biotechnology Associates) for 30 minutes in the dark. Directly labeled monoclonals were: FITC-labeled Vß11, Vß21.3, Vß13.6, Vß14, Vß16, Vß17, Vß20 and Vß22 and PE-labeled Vß9 and Vß23 (Immunotech). Cells were fixed with 1% paraformaldehyde, washed with FACSFlow buffer (Becton Dickinson) and analyzed using a Becton Dickinson flow cytometer.

EXAMPLES

CAP1 Substituted Peptides

Several factors were considered in deciding which positions to examine for effects on T cell activity. Sequencing and mapping experiments have defined a binding motif in which position 2 and the C-terminal (position 9 or 10) are critical for peptide presentation by HLA-A2

(for review, see 31). In addition, Tyr at position 1 has been identified as an effective secondary anchor (20, 32). Since the CEA peptide CAP1 already has the preferred amino acids at these three positions these residues were not altered. Instead, we focused attention on residues predicted to interact with the TCR in the hope of finding analogs that would stimulate human CAP1-specific cytotoxic T cells. X-ray crystallographic studies of several peptides bound to soluble HLA-A2 suggest that all binding peptides assume a common conformation in the peptide binding groove (33). When five model peptides were examined, residues 5 through 8 protrude away from the binding groove and are potentially available for binding to a TCR. Therefore a panel of 80 CAP1 analog peptides was produced in which the residues at positions 5 through 8 (p5-p8) were synthesized with each of the 20 natural amino acids. The peptides are designated CAP1-pAA, where p refers to the position in the peptide and AA refers to the replacement amino acid, using the single letter amino acid code; i.e., CAP1-6D in which position 6 is occupied by aspartic acid.

Enhanced CTL Sensitivity of Targets to CAP1-6D Analog

The effects of these amino acid substitutions on potential TCR recognition was studied using a CAP1 specific, HLA-A2 restricted human CTL line designated T-Vac8. Briefly, T-Vac8 was generated as described in Materials and Methods by in vitro peptide stimulation of PBMC from a patient that had been administered rV-CEA. For initial screening, T-Vac8 was used in a cytotoxicity assay to measure ^{111}In release from labeled C1R-A2 cells

incubated with each member of the peptide panel (at three peptide concentrations). Spontaneous release from the targets (in the absence of T-Vac8) was determined for each individual peptide.

The results are presented in Figure 1A through 1D. Of the 80 single amino acid substitutions, most failed to activate cytotoxicity of T-Vac8. However, six independent substitutions preserved reactivity. At position 5, three analogs CAP1-5F, CAP1-5I and CAP1-5S provided stimulation, albeit at reduced levels compared to CAP1 itself. At position 6 the substitutions CAP1-6C and CAP1-6D activated T-Vac8 cytotoxicity and seemed to be equal to or better than CAP1 since they were more active at the intermediate (0.1 μg/ml) peptide concentration. At position 7 analog CAP1-7I also appeared to be active. Finally, at position 8, no analogs were able to sensitize targets to lysis by T-Vac8. The two most active analogs (CAP1-6D and CAP1-7I) were then analyzed in detail, omitting CAP1-6C due to concern for disulfide formation under oxidizing conditions.

Purer preparations (90-96% pure) of native CAP1 and the analogs CAP1-6D and CAP1-71 were synthesized and compared in a CTL assay over a wider range of peptide concentrations, using two different cell lines as targets (Figure 2A and 2B). Employing T2 cells analog CAP1-6D was at least 10^2 times more effective than native CAP1. CAP1-6D lytic activity was at 1/2 maximum at 10^{-4} μg/ml (Figure 2A). In contrast, the CAP1-71 analog and the native CAP1 sequence were comparable with each other over the entire range of peptide titration and showed half maximal lysis at 10^{-2} μg/ml. Employing the C1R-A2 cells

as targets, CAP1-6D was similarly between 10^2 and 10^3 more effective in mediating lysis than CAP1 (Figure 2B).

The CAP1-6D peptide was also tested using a second CEA-specific T cell line, T-Vac24 (11). This line was generated from rV-CEA post vaccination PBMC of a different carcinoma patient by in vitro stimulation with the native CAP1 peptide; in contrast to predominantly CD8+ T-Vac8, T-Vac24 has a high percentage of CD4+CD8+ double positive cells (11). In a 4 hr ^{111}In release assay employing T-Vac24, CAP1-6D was slightly more effective (30% lysis) than the native CAP1 sequence (20% lysis); although the differences were not as pronounced as with T-Vac8, the increased sensitivity to the analog was seen in three separate experiments. The analog peptide clearly engaged the lytic apparatus of a second CAP1 specific CTL.

Analogs and Native Peptide Show Identical Presentation by HLA-A2

The increased effectiveness of CAP1-6D in CTL assays could be due to better presentation by the target. The most active CAP1 analogs were tested for binding to HLA-A2 by measuring cell surface HLA-A2 in the transport-defective human cell line T2. When compared over a 4-log range of concentrations, native CAP1 and the two analogs CAP1-6D and CAP1-7I all presented equally on T2 cells (Figure 3). In addition, dissociation experiments indicate that the HLA-A2 complexes that form with the 3 peptides show no appreciable differences in stability (Figure 3 - insert). When peptide-pulsed T2 cells were washed free of unbound peptide, the half lives of cell surface peptide-A2 complexes were 12.5 hrs (CAP1),

9.7 hrs (CAP1-6D), and 10.8 hrs (CAP1-71). If anything, the complex formed with the agonist peptide seems slightly less stable. Since there are no differences in binding to HLA-A2, the improved effectiveness of CAP1-6D in the CTL assays appears to be due to better engagement by the T cell receptor, a behavior characteristic of an enhancer agonist peptide.

Human CTL Generated With CAP1-6D Also Recognize Native CAP1

The CAP1-6D agonist might be useful in both experimental and clinical applications if it can stimulate growth of CEA-specific CTL from patients with established carcinomas. In one experiment, post rV-CEA immunization PBMC from cancer patient Vac8 (the same rV-CEA patient from whom T-Vac8 CTL were established) were stimulated in vitro with CAP1-6D and after 5 rounds of stimulation were assayed for CTL activity against targets coated with CAP1 or CAP1-6D. This new line demonstrated peptide-dependent cytotoxic activity against target cells coated with either CAP1-6D or native CAP1 (Table 1).

Post immunization PBMC from patients Vac8 and Vac24 were already shown to produce CTL activity when stimulated with CAP1 while preimmunization PBMC were negative (11, 34). Moreover, previous attempts to stimulate CTL activity from healthy, non-immunized donors with the CAP1 peptide were unsuccessful. To test if the agonist peptide is indeed more immunogenic than native CAP1 we attempted to generate CTL from healthy, non-immunized donors using CAP1-6D. HLA-A2+ PBMC from apparently healthy individuals were stimulated in vitro

either with CAP1 or the CAP1-6D agonist. After 4 cycles of in vitro stimulation, cell lines were assayed for specificity against C1R-A2 cells pulsed with either CAP1 or CAP1-6D.

While stimulations with CAP1 or the CAP1-6D peptide produced T cell lines, peptide specific lysis was only obtained in the lines generated with CAP1-6D. Two independent T cell lines from different donors were derived using CAP1-6D and were designated T-N1 and T-N2 (Figure 4A and Figure 4B respectively). Both CTL lines lyse C1R-A2 targets pulsed with native CAP1 peptide. However, more efficient lysis is obtained using the CAP1-6D agonist. T-N1 CTL recognizes CAP1-6D at a 3-10 fold lower peptide concentration than CAP1 and T-N2 recognizes the agonist 100 fold better than CAP1. In contrast, attempts to generate a CTL cell line from normal donors by stimulation with CAP1 resulted in lines with no peptide-dependent lysis and loss of the lines in early stimulation cycles. Thus the attempts to generate T cell lines using the two peptides demonstrated the ability of CAP1-6D to act as an agonist not only at the effector stage, in the lysis of targets, but also in selecting T cells that are presumably in low precursor frequencies.

To determine whether CTL established with the agonist could be maintained on the native CAP1 sequence, T-N1 was cultured for 5 cycles as described using CAP1-6D, then divided into duplicate cultures maintained on the agonist or on CAP1. T-N1 continued to grow when stimulated with either peptide and responded to both peptides in CTL assays. Phenotypic analysis of the TCR usage in T-N1 indicates that the majority of cells (71%)

utilize Vß12, with a minor population that utilize Vß5.3 (Table 2). The same pattern of TCR Vß usage was observed after switching the cells to CAP1 for 5 more stimulation cycles. This Vß usage pattern was distinct from that of T-Vac8. These data indicate that the agonist can select T cells that are probably in low precursor frequency but that once selected, such CTL could be maintained with the native CAP1.

CTL Generated With CAP1-6D Specifically Lysed CEA^+,$HLA\text{-}A2^+$ Tumor Cells

Studies were conducted to determine the ability of CTL generated with the enhancer agonist to lyse human tumor cells endogenously expressing CEA. T-N1 and T-N2 were tested against a panel of tumor cells that are $CEA^+/A2^+$ (SW480 and SW1463), CEA+/$A2^-$ (SW1116) or $CEA^-/A2^+$ (CaOV3 and SKmel24). A T cell line (T-N2) from the normal donor was tested for the ability to lyse tumor targets endogenously expressing CEA. T-N2 CTL generated with the agonist lysed tumor cells expressing both CEA and HLA-A2 while exhibiting no titratable lysis of $CEA^-/A2^+$ SKmel24 melanoma cells (Figure 5A). No significant lysis of K562 was observed. In contrast, cell lines generated by stimulation with native CAP1 showed no detectable antitumor activity (Figure 5B). The HLA-A2.1 restriction of the T-N2 response to CEA positive tumor targets was further demonstrated by the specific lysis of a CEA positive HLA-A2.1 negative tumor cell, SW837 after infection with a vaccinia-A2.1 construct (rV-A2.1). No lysis was observed when SW837 targets were infected with the control wild type vaccinia without the A2.1 transgene

(Figure 6).

The ability of a CTL line (T-N1) derived from a second donor to kill carcinoma targets expressing endogenous CEA is shown in Figure 7A and 7B. T-N1 specifically lysed SW480 tumor cells. This is dramatically enhanced to 79% lysis by pretreatment of the tumor cells with IFN-γ, a treatment that increases the cell surface density of both HLA-A2 and CEA. The specificity of T-N1 killing is demonstrated by its inability to lyse $CEA^-/A2^+$ tumors such as the ovarian derived tumor CaOV3, the melanoma tumor SKmel24, or the NK target K562. Finally, restriction by HLA-A2 is demonstrated by the failure of T-N1 to lyse $CEA^+/A2^-$ SW1116 tumor cells (Figure 7A), even after IFN-γ treatment (Figure 7B).

Table 1: CTL generated by stimulation with the CAP1-6D analog from PBMC of an HLA-A2 patient immunized with rVCEA

	% Lysis		
Effector/target ratio	no peptide	CAP1	CAP1-6D
25:1	10%	41%	40%
6.25:1	0.5%	38%	46%

T cells were assayed after 5 in vitro stimulations. Cytotoxic activity was determined in 4 hour release assay with peptide at 25 μg/ml.

Table 2: TCR usage of CTL line established on CAP1-6D agonist

TCR usage[a]	T-N1[b]		T-N1[c]	
	% positive	MFI	% positive	MFI
Vβ12	71	83	70	83
Vβ5.3	18	47	20	57
Vβ3.1	6	48	8	46
Vβ8	3	30	6	26
Vβ13.6	2	19	3	39
Vβ12.1	3	43	3	40

[a] Determined by FACS analysis using a panel of 19 Vβ and 2 Vα antibodies (see Materials and Methods). Only positively staining antibodies are shown.

[b] CTL line selected and maintained on agonist CAP1-6D as described in the Materials and Methods section.

[c] CTL line selected on agonist CAP1-6D for 5 stimulation cycles, and maintained on CAP1 for an additional 10 cycles.

This invention has been described in detail including preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention.

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We claim:

1. A peptide comprising an agonist of a native sequence:

YLSGANLNL (Seq. ID No: 1)
| |
123456789

2. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 6 from Seq. ID No: 1.

3. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 7 from Seq. ID No: 1.

4. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 6 and position 7 from Seq. ID No: 1.

5. The peptide according to claim 1 containing YLSGADLNL (Seq. ID No: 2), YLSGADINL (Seq. ID No: 3), YLSGANINL (Seq. ID No: 4), YLSGACLNL (Seq. ID No: 5), or combination thereof.

6. A peptide consisting of the amino acid sequence YLSGADLNL (Seq. ID No: 2), YLSGADINL (Seq. ID No: 3), or YLSGANINL (Seq. ID No: 4), YLSGACLNL (Seq. ID No: 5).

7. A pharmaceutical composition comprising the peptide according to claim 1 and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition according to claim 7 further comprising an immunostimulatory molecule.

9. The pharmaceutical composition according to claim 8 wherein the immunostimulatory molecule is selected from the gproup consisting of IL-2, B7.1, B7.2, ICAM-1,

LFA-3, CD72, GM-CSF, TNFα, INFγ, IL-12, IL-6 and combinations thereof.

10. The pharmaceutical composition according to claim 7 further comprising an HLA class I molecule or a cell expressing an HLA class I molecule.

11. The pharmaceutical composition according to claim 7 further comprising a chemotherapeutic drug, antibiotic, antiviral drug, antifungal drug, or cyclophosphamide.

12. The pharmaceutical composition according to claim 7 further comprising an adjuvant.

13. The pharmaceutical composition according to claim 12 wherein the adjuvant is selected from the group consisting of alum, incomplete Freund's adjuvant, QS21, and Ribi Detox™.

14. A peptide-immunoglobulin conjugate comprising the peptide according to claim 1 and an immunoglobulin molecule.

15. The pharmaceutical composition according to claim 7 wherein the peptide is incorporated into a liposome.

16. A peptide-carrier molecule conjugate comprising the peptide according to claim 1 conjugated to a carrier molecule.

17. The peptide-carrier molecule conjugate according to claim 15 wherein the carrier molecule is selected from the group consisting of influenza peptide, tetanus toxoid, tetanus toxoid-CD4 epitope, Pseudomonas exotoxin A, poly-L-lysine, a lipid tail and an endoplasmic reticulum signal sequence.

18. A kit comprising the agonist peptide

according to claim 1 and a vector comprising a nucleic acid sequence encoding CEA.

19. The kit according to claim 18 further comprising an immunostimulatory molecule.

20. An isolated DNA comprising a nucleotide sequence encoding the peptide according to claim 1 or variants thereof.

21. An isolated DNA encoding a peptide containing Seq. ID No: 2, Seq. ID No: 3, Seq. ID No: 4, Seq. ID No: 5, or combinations thereof.

22. An isolated DNA comprising a nucleotide sequence of SEQ. ID No: 7 or 8.

23. A vector comprising the DNA of claims 20, 21 or 22.

24. The vector according to claim 23 wherein the vector is an E. coli plasmid, a Listeria vector, an orthopox virus, avipox virus, capripox virus, suipox virus, vaccinia virus, baculovirus, human adenovirus, SV40 or bovine papilloma virus.

25. The vector according to claims 23 or 24 further comprising a nucleotide sequence encoding at least one HLA class I molecule.

26. A host cell comprising the vector according to claim 23.

27. The host cell according to claim 26 wherein the host cell additionally expresses an HLA class I molecule.

28. The host cell according to claim 26 wherein the host cell is an antigen presenting cell.

29. The host cell according to claim 28 wherein the host cell is a dendritic cell.

30. A method for treating a host having a tumor expressing CEA or epitope thereof comprising introducing cytotoxic T lymphocytes specific for CEA or epitope thereof to the host and at a periodic interval thereafter introducing to the host an agonist peptide according to claim 1.

31. The method according to claim 30 wherein the peptide is selected from the group consisting of Seq ID Nos: 2, 3, 4, 5 or combination thereof.

32. A method of inhibiting a CEA epitope-expressing carcinoma cells in a patient comprising administering to said patient an effective amount of the peptide according to claim 1.

33. The method according to claim 32 further comprising administration of an immunostimulatory molecule.

34. The method according to claim 33 wherein the immunostimulatory molecule is selected from the group consisting of IL-2, B7.1, B7.2, ICAM-1, LFA-3, CD72, GM-CSF, TNFα, INFγ, IL-12, IL-6 and combinations thereof.

35. The method according to claim 32 further comprising administration of an adjuvant.

36. The method according to claim 32 wherein the carcinoma cell is gastrointestinal, breast, pancreatic, bladder, ovarian, lung, or prostate carcinoma cells.

37. The method according to claim 32 further comprising the administration of a vector comprising the gene encoding CEA.

38. A method of inhibiting or killing CEA epitope-expressing carcinoma cells comprising:

A) generating CEA epitope or agonist peptide-specific cytotoxic T lymphocytes *in vitro* by stimulation of lymphocytes from a source with an effective amount of an agonist peptide according to claim 1 alone or in combination with an immunostimulatory molecule; and

B) adoptively transferring the CEA epitope or agonist peptide-specific cytotoxic T lymphocytes alone or in combination with the agonist peptide into a mammal in an amount sufficient to inhibit or kill the CEA epitope expressing carcinoma cells.

39. A method of inhibiting or killing CEA epitope-expressing carcinoma cells in a mammal comprising:

A) generating CEA epitope or agonist peptide-specific cytotoxic T lymphocytes *in vivo* by administration of an effective amount of a agonist peptide according to claim 1, an effective amount of a vector comprising a nucleic acid sequence encoding CEA or agonist peptide pulsed antigen presenting cells; and

B) at a periodic interval providing the agonist peptide according to claim 1 alone or in combination with an adjuvant;

wherein the CEA epitope or agonist peptide-specific cytotoxic T lymphocytes so generated inhibit or kill CEA epitope-expressing carcinoma cells.

40. A peptide comprising an antagonist of a

native sequence: YLSGANLNL (Seq. ID No: 1) wherein the antagonist varies at at least one amino acid position from SEQ. ID No: 1 and the antagonist inhibits CEA-specific immune responses.

41. A pharmaceutical composition comprising the peptide according to claim 36 and a pharmaceutically acceptable carrier.

42. A method of inhibiting CEA-specific immune responses comprising administration of the peptide according to claim 40 in an amount effective to inhibit the CEA-specific immune responses.

43. The method according to claim 42 wherein cytotoxic T lymphocytes specific for CEA or epitopes thereof are inhibited.

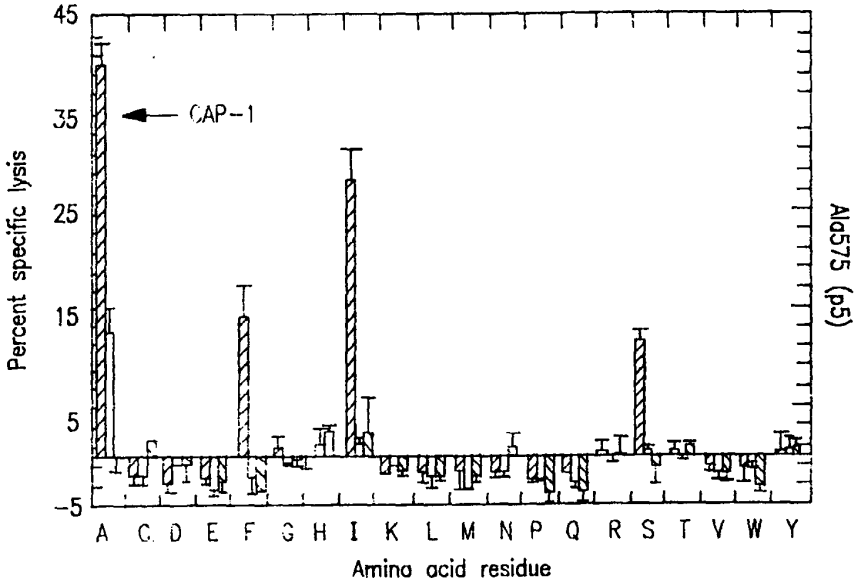
45
35
25
15
5
-5
CAP-1
Percent specific lysis
A C D E F G H I K L M N P Q R S T V W Y
Amino acid residue
Ala575 (p5)

FIG. IA

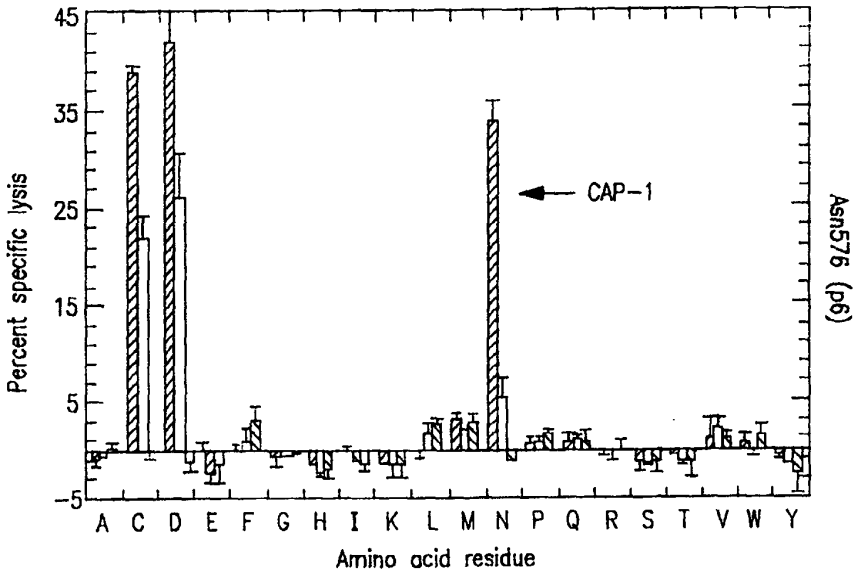
45
35
25
15
5
-5
CAP-1
Percent specific lysis
A C D E F G H I K L M N P Q R S T V W Y
Amino acid residue
Asn576 (p6)

FIG. IB

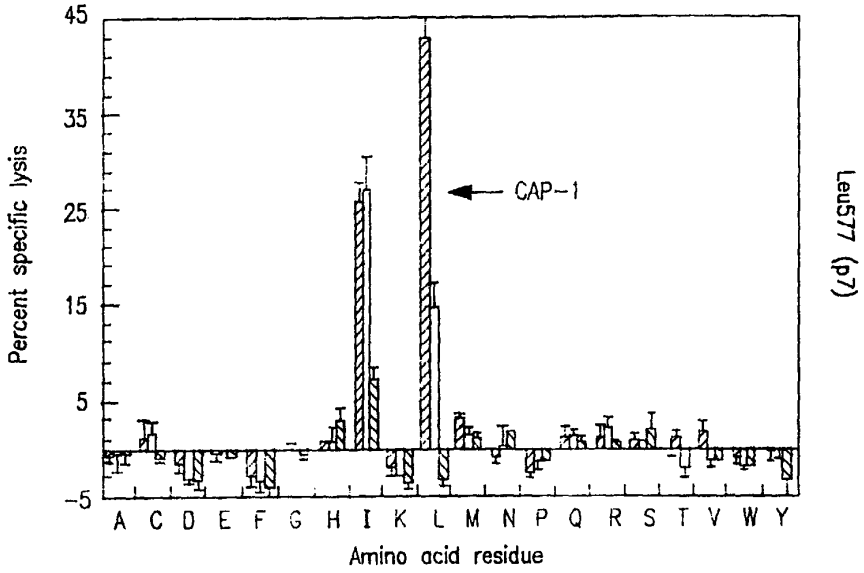
45
35
25
15
5
-5
Percent specific lysis
CAP-1
Leu577 (p7)
A C D E F G H I K L M N P Q R S T V W Y
Amino acid residue

FIG. IC

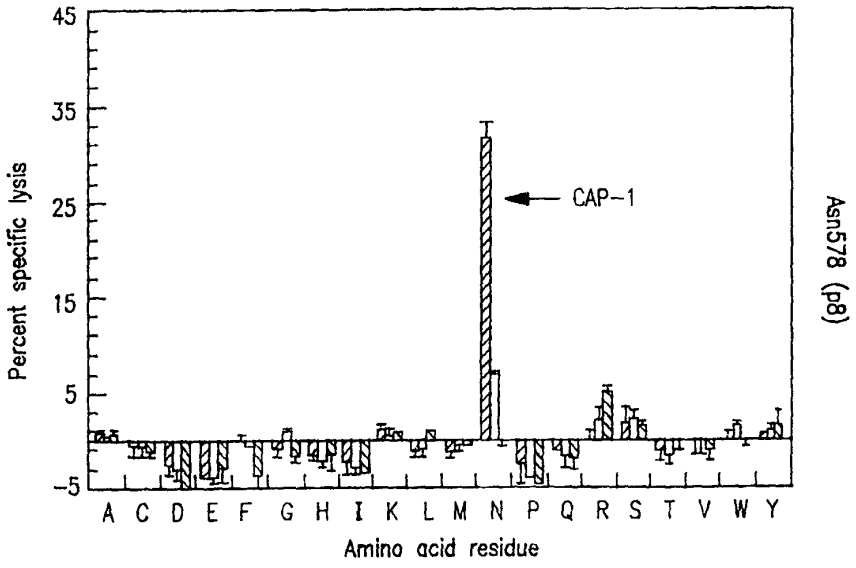
45
35
25
15
5
-5
Percent specific lysis
CAP-1
Asn578 (p8)
A C D E F G H I K L M N P Q R S T V W Y
Amino acid residue

FIG. ID

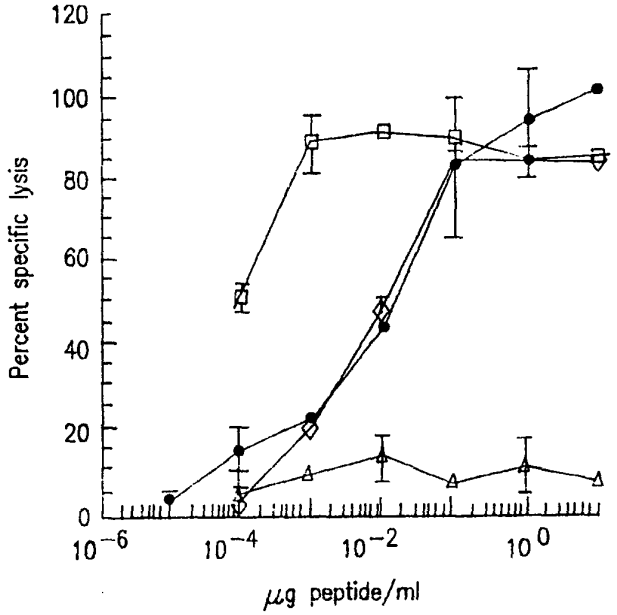
120
100
80
60
40
20
0
Percent specific lysis
10^{-6}
10^{-4}
10^{-2}
10^{0}
μg peptide/ml

FIG. 2A

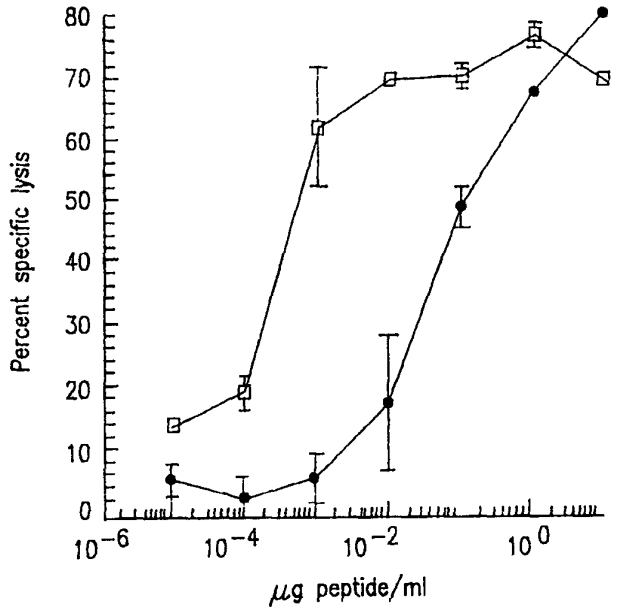
80
70
60
50
40
30
20
10
0
Percent specific lysis
10^{-6}
10^{-4}
10^{-2}
10^{0}
μg peptide/ml

FIG. 2B

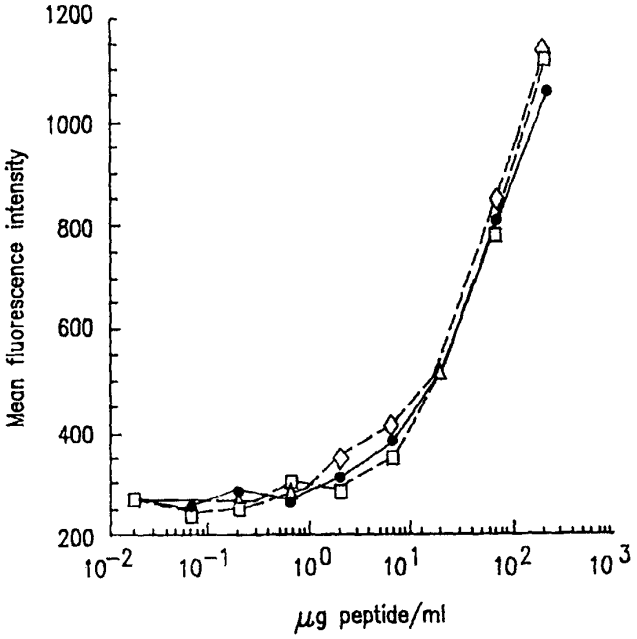
1200
1000
800
600
400
200
Mean fluorescence intensity
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
μg peptide/ml

FIG. 3A

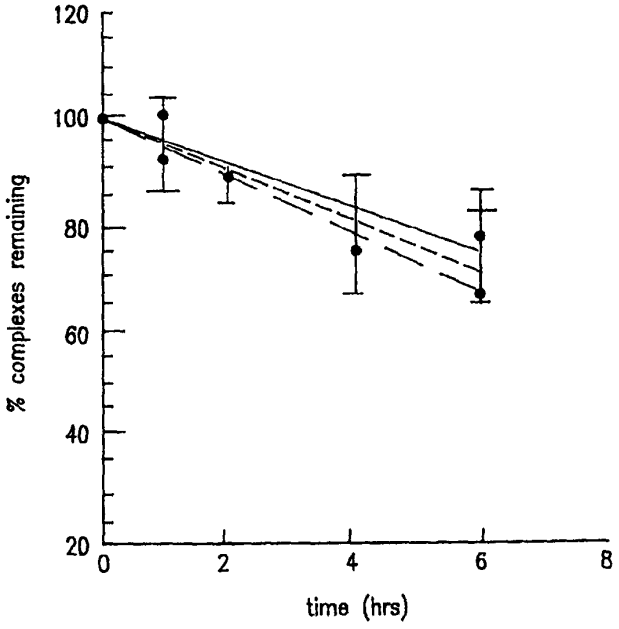
120
100
80
60
40
20
% complexes remaining
0
2
4
6
8
time (hrs)

FIG. 3B

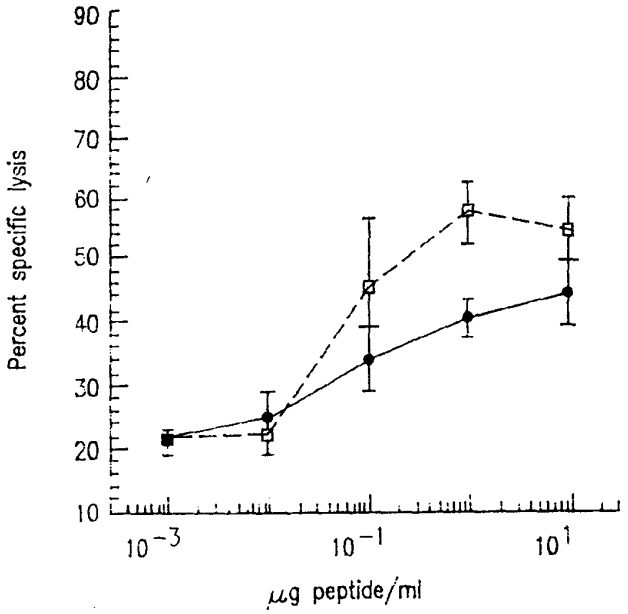
Percent specific lysis
90
80
70
60
50
40
30
20
10
10^{-3}
10^{-1}
10^{1}
μg peptide/ml

FIG. 4A

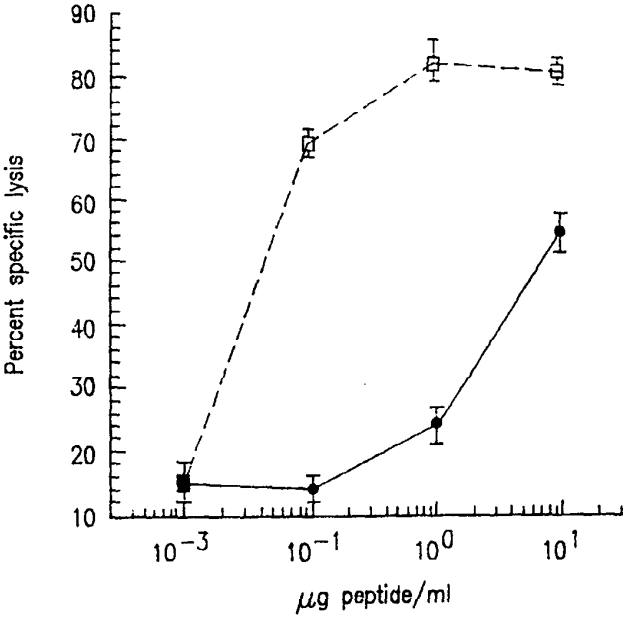
Percent specific lysis
90
80
70
60
50
40
30
20
10
10^{-3}
10^{-1}
10^{0}
10^{1}
μg peptide/ml

FIG. 4B

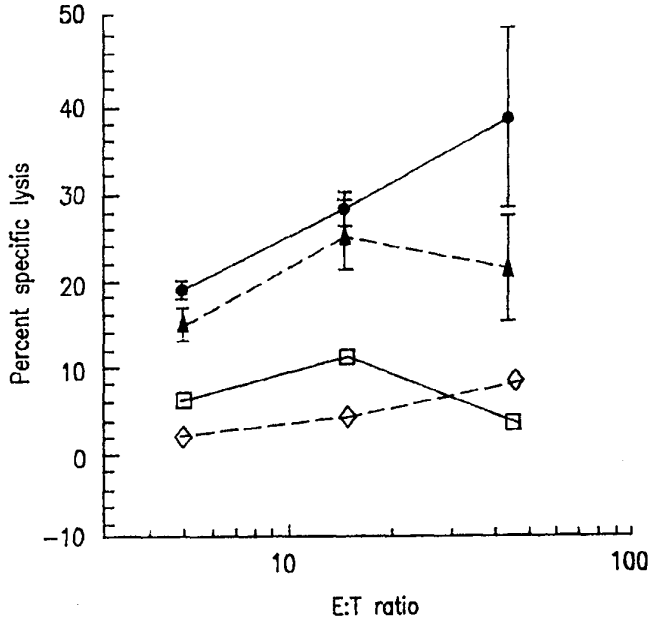
50
40
30
20
10
0
-10
Percent specific lysis
10
100
E:T ratio

FIG. 5A

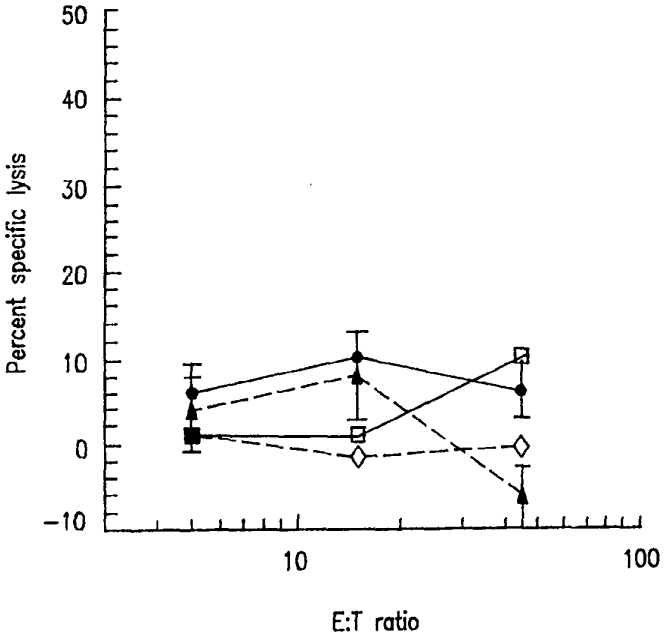
50
40
30
20
10
0
-10
Percent specific lysis
10
100
E:T ratio

FIG. 5B

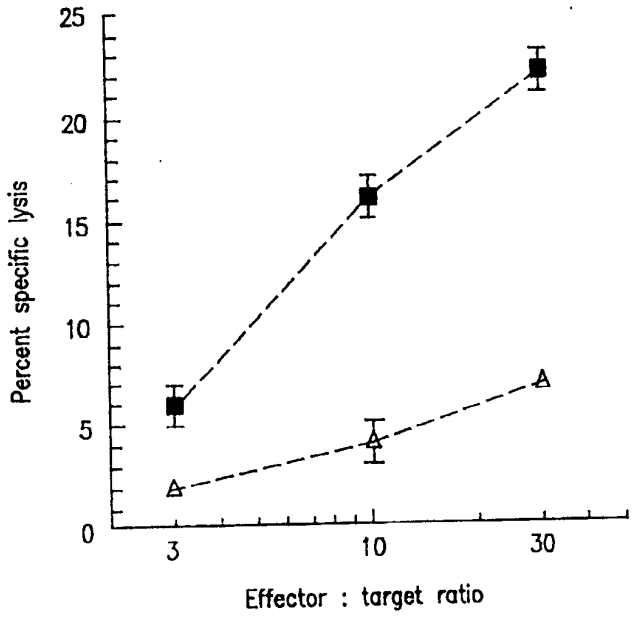
25
20
15
10
5
0
Percent specific lysis
3
10
30
Effector : target ratio

FIG. 6

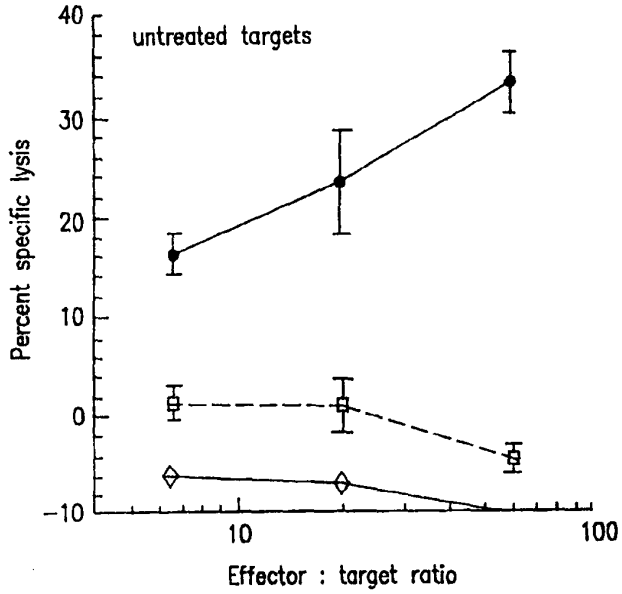
untreated targets
Percent specific lysis
40
30
20
10
0
-10
10
100
Effector : target ratio

FIG. 7A

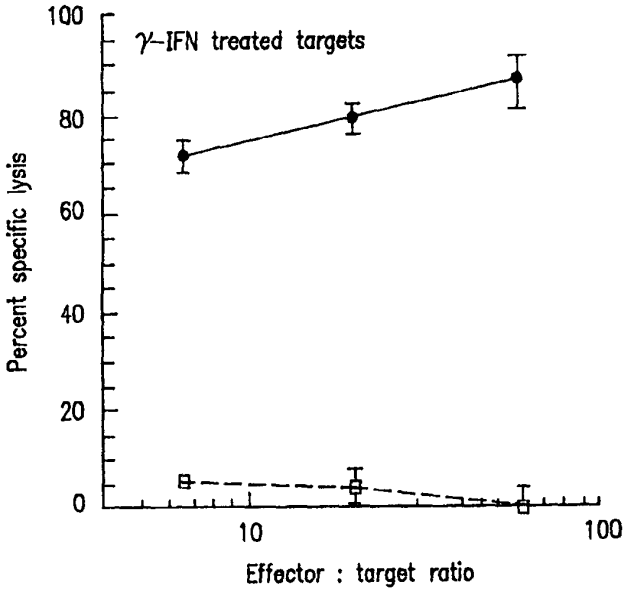
γ-IFN treated targets
Percent specific lysis
100
80
60
40
20
0
10
100
Effector : target ratio

FIG. 7B

INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 98/19794

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 A61K38/17 A61K47/00 A61K35/14 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZAREMBA S. ET AL.: "Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen" CANCER RESEARCH, vol. 57, no. 20, 15 October 1997, pages 4570-4577, XP002096107 see the whole document	1-7
A	WO 96 26271 A (THERION BIOLOGICS CORP (US); US GOVERNMENT; SCHLOM; PANICALI; TSANG) 29 August 1996 see abstract see page 4, line 1 - page 6, line 17 see page 12, line 1 - page 15, line 13 see page 62 - page 67; claims	1,7-9, 12,13, 15,18, 19,30, 32-39

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1999	25/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/US 98/19794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	TSANG K.Y. ET AL.: "Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant Vaccinia-CEA vaccine" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 87, no. 13, 5 July 1995, pages 982-990, XP000578019 cited in the application see the whole document	1,7-9, 12,13, 15,18, 19,30, 32-39
A	WO 92 19266 A (US GOVERNMENT; SCHLOM J.; KANTOR J.) 12 November 1992 see page 10, line 14 - page 11, line 18 see page 23, line 25 - page 26, line 5; examples 10,11	8,9,11
A	CHEN Y.-Z. ET AL.: "Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide" THE JOURNAL OF IMMUNOLOGY, vol. 157, 1996, pages 3783-3790, XP002096108 cited in the application see abstract	40
A	JAMESON S.C. ET AL.: "T cell receptor antagonists and partial agonists" IMMUNITY, vol. 2, no. 1, January 1995, pages 1-11, XP000600494 cited in the application see page 8 - page 9	40

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 19794

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 30-39, 42, 43 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 98/19794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9626271 A	29-08-1996	AU 5024096 A	11-09-1996
		CA 2213451 A	29-08-1996
		EP 0811062 A	10-12-1997
WO 9219266 A	12-11-1992	AU 674492 B	02-01-1997
		AU 2006092 A	21-12-1992
		CA 2102623 A	07-11-1992
		EP 0584266 A	02-03-1994
		JP 6508025 T	14-09-1994
		US 5698530 A	16-12-1997

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
Morgan & Finnegan, L.L.P.
Attn. FEILER, W.
345 Park Avenue
New York, New York 10154
UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 2026-4266PC	Date of mailing (day/month/year) 25/03/1999
International application No. PCT/US 98/ 19794	FOR FURTHER ACTION See paragraphs 1 and 4 below
Applicant THE GOVERNMENT OF THE UNITED STATES OF A. ..et al.	International filing date (day/month/year) 22/09/1998

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2026-4266PC ATTY KHS
DUE MAY 25, 1999
1 MO. CALL-UP MAY 25, 1999
REPLY RECEIVED BY HJC

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mireille Claudepierre

CASE 2026-4266 PC ATTY KHS
DUE JUNE 25, 1999
1 MO. CALL-UP MAY 25, 1999
BY HJC

Form PCT/ISA/220 (July 1998)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
2026-4266PC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/ 19794	22/09/1998	10/10/1997
Applicant		
THE GOVERNMENT OF THE UNITED STATES OF A. ..et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   AGONIST AND ANTAGONIST PEPTIDES OF CARCINOEMBRYONIC ANTIGEN (CEA)

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 19794

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 30-39, 42, 43 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/19794

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 A61K38/17 A61K47/00 A61K35/14 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZAREMBA S. ET AL.: "Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen" CANCER RESEARCH, vol. 57, no. 20, 15 October 1997, pages 4570-4577, XP002096107 see the whole document ---	1-7
A	WO 96 26271 A (THERION BIOLOGICS CORP (US); US GOVERNMENT; SCHLOM; PANICALI; TSANG) 29 August 1996 see abstract see page 4, line 1 - page 6, line 17 see page 12, line 1 - page 15, line 13 see page 62 - page 67; claims --- -/--	1,7-9, 12,13, 15,18, 19,30, 32-39

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1999	25/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/19794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	TSANG K.Y. ET AL.: "Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant Vaccinia-CEA vaccine" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 87, no. 13, 5 July 1995, pages 982-990, XP000578019 cited in the application see the whole document ---	1,7-9, 12,13, 15,18, 19,30, 32-39
A	WO 92 19266 A (US GOVERNMENT; SCHLOM J.; KANTOR J.) 12 November 1992 see page 10, line 14 - page 11, line 18 see page 23, line 25 - page 26, line 5; examples 10,11 ---	8,9,11
A	CHEN Y.-Z. ET AL.: "Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide" THE JOURNAL OF IMMUNOLOGY, vol. 157, 1996, pages 3783-3790, XP002096108 cited in the application see abstract ---	40
A	JAMESON S.C. ET AL.: "T cell receptor antagonists and partial agonists" IMMUNITY, vol. 2, no. 1. January 1995, pages 1-11. XP000600494 cited in the application see page 8 - page 9 -----	40

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/19794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9626271 A	29-08-1996	AU 5024096 A	11-09-1996
		CA 2213451 A	29-08-1996
		EP 0811062 A	10-12-1997
WO 9219266 A	12-11-1992	AU 674492 B	02-01-1997
		AU 2006092 A	21-12-1992
		CA 2102623 A	07-11-1992
		EP 0584266 A	02-03-1994
		JP 6508025 T	14-09-1994
		US 5698530 A	16-12-1997

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
2026-4266PC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/ 19794	22/09/1998	10/10/1997
Applicant		
THE GOVERNMENT OF THE UNITED STATES OF A. ..et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] *furnished subsequently to this Authority in computer readble form.*

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   AGONIST AND ANTAGONIST PEPTIDES OF CARCINOEMBRYONIC ANTIGEN (CEA)

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 19794

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 30-39, 42, 43 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/19794

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 A61K38/17 A61K47/00 A61K35/14
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZAREMBA S. ET AL.: "Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen" CANCER RESEARCH, vol. 57, no. 20, 15 October 1997, pages 4570-4577, XP002096107 see the whole document ---	1-7
A	WO 96 26271 A (THERION BIOLOGICS CORP (US); US GOVERNMENT; SCHLOM; PANICALI; TSANG) 29 August 1996 see abstract see page 4, line 1 - page 6, line 17 see page 12, line 1 - page 15, line 13 see page 62 - page 67; claims --- -/--	1,7-9, 12,13, 15,18, 19,30, 32-39

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1999	25/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	TSANG K.Y. ET AL.: "Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant Vaccinia-CEA vaccine" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 87, no. 13, 5 July 1995, pages 982-990, XP000578019 cited in the application see the whole document	1,7-9, 12,13, 15,18, 19,30, 32-39
A	WO 92 19266 A (US GOVERNMENT; SCHLOM J.; KANTOR J.) 12 November 1992 see page 10, line 14 - page 11, line 18 see page 23, line 25 - page 26, line 5; examples 10,11	8,9,11
A	CHEN Y.-Z. ET AL.: "Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide" THE JOURNAL OF IMMUNOLOGY, vol. 157, 1996, pages 3783-3790, XP002096108 cited in the application see abstract	40
A	JAMESON S.C. ET AL.: "T cell receptor antagonists and partial agonists" IMMUNITY, vol. 2, no. 1, January 1995, pages 1-11, XP000600494 cited in the application see page 8 - page 9	40

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/19794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9626271 A	29-08-1996	AU 5024096 A	11-09-1996
		CA 2213451 A	29-08-1996
		EP 0811062 A	10-12-1997
WO 9219266 A	12-11-1992	AU 674492 B	02-01-1997
		AU 2006092 A	21-12-1992
		CA 2102623 A	07-11-1992
		EP 0584266 A	02-03-1994
		JP 6508025 T	14-09-1994
		US 5698530 A	16-12-1997

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

FEILER, William S.
Morgan & Finnegan, L.L.P.
345 Park Avenue
New York, New York 10154
ETATS-UNIS D'AMERIQUE

EPO/PCT Rec'd 06 APR 2000

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	20.01.00
Applicant's or agent's file reference 2026-4266PC	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/19794	22/09/1998	10/10/1997

Applicant
THE GOVERNMENT OF THE UNITED STATES OF A. .. et al

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

M.H

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

17

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2026-4266PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/19794	22/09/1998	10/10/1997
International Patent Classification (IPC) or national classification and IPC C12N15/12		
Applicant THE GOVERNMENT OF THE UNITED STATES OF A. .. et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/04/1999	20.01.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Paresce, D Telephone No. +49 89 2399 8995

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/19794

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-12,14-52	as originally filed			
13	as received on	29/11/1999	with letter of	24/11/1999

Claims, No.:

1-45	as received on	29/11/1999	with letter of	24/11/1999

Drawings, sheets:

1/8-8/8	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-45
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-45
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-29, 40-41, 44-45
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1) Reference is made to the following document:

D1: WO 96 26271 A (THERION BIOLOGICS CORP (US); US GOVERNMENT; SCHLOM; PANICALI; TSANG) 29 August 1996

2) Novelty: Article 33(2) PCT

D1 describes methods for producing human cytotoxic T-cells specific for a carcinoma self associated antigen, in particular the carcinoembryonic antigen (CEA). The cytotoxic T-cells can be used to determine the epitopes of CEA that elicit a cytotoxic T-cell response. D1 discloses that peptide fragments of CEA protein were identified that would bind HLA class I molecule (p. 40, last paragraph). The binding of CEA peptides to HLA-A2 molecule was analysed by the upregulation of HLA-A2 expression of T2 cells (p. 35). These peptide fragments, rather than the whole CEA protein were used to stimulate cytotoxic T-cells (p. 13, last 2 paragraphs). The binding of the CEA peptides to HLA-A2 molecule and T2 binding is shown in Table 7, p. 47.
D1 also describes a recombinant DNA viral vector, preferably a pox virus, that contains a DNA segment encoding a CEA epitope linked to a promoter capable of expression in a host cell (see abstract). D1 describes methods of stimulating production of cytotoxic T-cells by introducing said recombinant pox virus vector into a host and then contacting the host with additional antigen at periodic intervals thereafter. The antigen can be formulated with an adjuvant, preferably RIBI Detox, QS21 or incomplete Freund's adjuvant (see claim 17), or in a liposomal formulation (see abstract). D1 discloses methods to treat a host having a tumour expressing CEA (claims 28-34).

The subject-matter of claims 1-45 has not been made available to the public by any of the available prior art documents and can therefore be regarded as novel.

3) Inventive Step: Article 33(3) PCT

The subject-matter of claims 1-45 cannot be derived from the available prior art in an obvious manner and therefore complies with the requirements of Article 33(3) PCT.

VIII. Certain observations on the international application

1) Clarity: Article 6 PCT

Article 6 PCT requires amongst other things that the claims, which define the matter for which protection is sought (i.e. the object of invention) be clear. This has to be interpreted as meaning not only that a claim from a technical point of view must be comprehensible, but also that it must define clearly the object of the invention, that is to say, it must indicate all the essential features thereof. The essential features are regarded as all features which are necessary to obtain the desired effect, or differently expressed, those features which are necessary to solve the technical problem with which the application is concerned. In other words, all technical features which enable the skilled person to put the claimed matter into practice without undue burden i.e. without experimentation or without application of inventive skill.

Claims 1 and 40 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The subject-matter of these claims is too imprecisely drafted and merely paraphrases the technical problem with which the application is concerned. The claims attempt to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result should be added.

From reading claims 1 and 40 it is apparent that one amino acid substitution at a non-MHC anchor position of SEQ ID NO:1 can have two very different results: in one case the resulting peptide may have "enhanced immunogenicity compared to the native sequence", in a second case the resulting peptide "inhibits CEA-specific immune responses". The technical features given, namely, "one amino acid substitution at a non-MHC anchor position of SEQ ID NO:1" are not sufficient to enable the skilled person to arrive at the desired result without experimentation or

without application of inventive skill. It is, in fact the very object of the present application to discover which of the many possible amino acid substitutions of SEQ ID NO:1 yield the desired result. The IPEA, therefore, is of the opinion that claims 1 and 40 do not meet the requirements of Article 6 PCT.

Claims 5, 21, 31 are not clear. These claims are directed to a peptide according to claim 1 containing a given sequence or "combination thereof". It is not clear what the term "combination thereof" refers to. Does this mean a combination of the different sequences or amino acids. How are the sequences to be "combined"?

2) **Objections under Article 6 PCT in combination with Article 5 PCT**

Claims 40-43 are not supported by the description as required by Article 6 PCT, as their scope is broader than justified by the description and drawings. The reasons therefor are the following:

Article 6 PCT requires the claims to be fully, i.e. formally and technically, supported by the description. In the present application, the peptides CAP1-6D and CAP1-6D, 7I are described. CAP1-6D is described as an agonist which enhances recognition of the peptide-MHC ligand by the T-cell receptor and produces greater effector function without increases in binding (present application, p. 24). CAP1-6D, 7I is also considered an "agonist" (p. 7). There is no real technical characterization, however, of any "antagonist" of CAP-1. There is not a single example in the present application in which a CEA peptide is used in to inhibit CEA-specific immune responses. There is no information given in the present application that would enable the skilled person to determine how to put the methods claimed in claims 42-43 into practice. There is, in fact, no indication that any of the claimed sequences are in fact suitable for use in the methods of these claims. The information given in the specification is insufficient to enable a skilled person to carry out the methods of claims 40-43 without undue experimentation or without application of inventive skill. Thus the IPEA is of the opinion that claims 40-43 that are not actually disclosed or technically well characterized in the sense of Article 5 PCT, are not supported by the description.

3) Additional comments

Claims 30-39, 42-43 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

For the assessment of the presently worded claims 30-39, 42-43 on the question whether they are industrially applicable no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPC, for example, does not recognise as industrially applicable claims to the use of a compound in medical treatment, but will allow however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

immunogen in a mammal, preferably a human. The composition may further comprise one or more other constituents to enhance the immune response which include but are not limited to immunostimulatory molecules such as interleukin 2, interleukin 6, interleukin 12, interferon gamma, tumor necrosis factor alpha, GM-CSF, B7.1, B7.2, ICAM-1, LFA-3, CD72, and cyclophosphamide.

The agonist peptide is administered to a mammal in an amount effective in generating a CEA specific immune response, preferably a cellular immune response. The efficacy of the agonist peptide as an immunogen may be determined by *in vivo* or *in vitro* parameters as are known in the art. These parameters include but are not limited to antigen specific cytotoxicity assays, regression of tumors expressing CEA or CEA epitopes, inhibition of cancer cells expressing CEA or CEA epitopes, production of cytokines and the like.

At least one or more agonist peptides may be administered in a dose of about 0.05 mg to about 10 mg per vaccination of the mammal, preferably about 0.1 mg to about 5 mg per vaccination. Several doses may be provided over a period of weeks as indicated. In one embodiment a dose is provided every month for 3 months. The agonist peptide may be administered alone or in combination with adjuvants, incorporated into liposomes (U.S. Patent Nos. 5,643,599; 5,464,630; 5,059,421; 4,885,172), with cytokines, biological response modifiers, or other reagents in the art that are known to enhance immune response. Adjuvants include but are not limited to RIBI Detox™, QS21, alum and incomplete Freund's adjuvant. In one embodiment, the agonist peptide is administered in

We claim:

1. A peptide comprising an agonist of a native sequence:

YLSGANLNL (Seq. ID No: 1)
| |
123456789;

wherein the agonist has at least one amino acid substitution at a non-MHC anchor position of SEQ ID NO: 1 and said agonist has enhanced immunogenicity compared to the native sequence.

2. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 6 from Seq. ID No: 1.

3. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 7 from Seq. ID No: 1.

4. The peptide according to claim 1 wherein the agonist varies in an amino acid substitution at position 6 and position 7 from Seq. ID No: 1.

5. The peptide according to claim 1 comprising an amino acid sequence selected from the group consisting of: YLSGADLNL (Seq. ID No: 2), YLSGADINL (Seq. ID No: 3), YLSGANINL (Seq. ID No: 4), YLSGACLNL (Seq. ID No: 5), and combinations thereof.

6. A peptide consisting of the amino acid sequence YLSGADLNL (Seq. ID No: 2), YLSGADINL (Seq. ID No: 3), or YLSGANINL (Seq. ID No: 4), YLSGACLNL (Seq. ID No: 5).

7. A pharmaceutical composition comprising at least one peptide according to any of claims 1 through 6 and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition according to claim 7 further comprising an immunostimulatory molecule.

9. The pharmaceutical composition according to claim 8 wherein the immunostimulatory molecule is selected from the group consisting of IL-2, B7.1, B7.2, ICAM-1,

LFA-3, CD72, GM-CSF, TNFα, INFγ, IL-12, IL-6 and combinations thereof.

10. The pharmaceutical composition according to claim 7 further comprising an HLA class I molecule or a cell expressing an HLA class I molecule.

11. The pharmaceutical composition according to claim 7 further comprising a chemotherapeutic drug, antibiotic, antiviral drug, antifungal drug, or cyclophosphamide.

12. The pharmaceutical composition according to claim 7 further comprising an adjuvant.

13. The pharmaceutical composition according to claim 12 wherein the adjuvant is selected from the group consisting of alum, incomplete Freund's adjuvant, QS21, and Ribi Detox™.

14. A peptide-immunoglobulin conjugate comprising the peptide according to any of claims 1 through 6 and an immunoglobulin molecule.

15. The pharmaceutical composition according to claim 7 wherein the peptide is incorporated into a liposome.

16. A peptide-carrier molecule conjugate comprising the peptide according to claim 1 conjugated to a carrier molecule.

17. The peptide-carrier molecule conjugate according to claim 16 wherein the carrier molecule is selected from the group consisting of influenza peptide, tetanus toxoid, tetanus toxoid-CD4 epitope, Pseudomonas exotoxin A, poly-L-lysine, a lipid tail and an endoplasmic reticulum signal sequence.

18. A kit comprising the agonist peptide

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according to claim 1 and a vector comprising a nucleic acid sequence encoding CEA.

19. The kit according to claim 18 further comprising an immunostimulatory molecule.

20. An isolated DNA comprising a nucleotide sequence encoding the peptide according to any of claims 1 through 6.

21. An isolated DNA encoding a peptide comprising the amino acid sequence selected from the group consisting of: Seq. ID No: 2, Seq. ID No: 3, Seq. ID No: 4, Seq. ID No: 5, and combinations thereof.

22. An isolated DNA comprising a nucleotide sequence of SEQ. ID No: 7 or 8.

23. A vector comprising the DNA of claims 20, 21 or 22.

24. The vector according to claim 23 wherein the vector is an E. coli plasmid, a Listeria vector, an orthopox virus, avipox virus, capripox virus, suipox virus, vaccinia virus, baculovirus, human adenovirus, SV40 or bovine papilloma virus.

25. The vector according to claims 23 or 24 further comprising a nucleotide sequence encoding at least one HLA class I molecule.

26. A host cell comprising the vector according to claim 23.

27. The host cell according to claim 26 wherein the host cell additionally expresses an HLA class I molecule.

28. The host cell according to claim 26 wherein the host cell is an antigen presenting cell.

29. The host cell according to claim 28 wherein the host cell is a dendritic cell.

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30. A method for treating a host having a tumor expressing CEA or epitope thereof comprising introducing cytotoxic T lymphocytes specific for CEA or epitope thereof to the host and at a periodic interval thereafter introducing to the host at least one agonist peptide according to any of claims 1 through 6.

31. The method according to claim 30 wherein the peptide comprises the amino acid sequence selected from the group consisting of: Seq ID Nos: 2, 3, 4, 5 and combinations thereof.

32. A method of inhibiting a CEA epitope-expressing carcinoma cells in a patient comprising administering to said patient an effective amount of the peptide according to any of claims 1 through 6.

33. The method according to claim 32 further comprising administration of at least one immunostimulatory molecule.

34. The method according to claim 33 wherein the immunostimulatory molecule is selected from the group consisting of IL-2, B7.1, B7.2, ICAM-1, LFA-3, CD72, GM-CSF, TNFα, INFγ, IL-12, IL-6 and combinations thereof.

35. The method according to claim 32 further comprising administration of an adjuvant.

36. The method according to claim 32 wherein the carcinoma cell is gastrointestinal, breast, pancreatic, bladder, ovarian, lung, or prostate carcinoma cells.

37. The method according to claim 32 further comprising the administration of a vector comprising the gene encoding CEA.

38. A method of inhibiting or killing CEA epitope-expressing carcinoma cells comprising:

A) generating CEA epitope or agonist peptide-specific cytotoxic T lymphocytes *in vitro* by stimulation of lymphocytes from a source with an effective amount of an agonist peptide according to any of claims 1 through 6 alone or in combination with an immunostimulatory molecule; and

B) adoptively transferring the CEA epitope or agonist peptide-specific cytotoxic T lymphocytes alone or in combination with the agonist peptide into a mammal in an amount sufficient to inhibit or kill the CEA epitope expressing carcinoma cells.

39. A method of inhibiting or killing CEA epitope-expressing carcinoma cells in a mammal comprising:

A) generating CEA epitope or agonist peptide-specific cytotoxic T lymphocytes *in vivo* by administration of an effective amount of a agonist peptide according to any of claims 1 through 6, an effective amount of a vector comprising a nucleic acid sequence encoding CEA or agonist peptide pulsed antigen presenting cells; and

B) at a periodic interval providing the agonist peptide according to any of claims 1 through 6 alone or in combination with an adjuvant;

wherein the CEA epitope or agonist peptide-specific cytotoxic T lymphocytes so generated inhibit or kill CEA epitope-expressing carcinoma cells.

40. A peptide comprising an antagonist of a

native sequence: YLSGANLNL (Seq. ID No: 1) wherein the antagonist has at least one amino acid substitution at a non-MHC anchor position of SEQ. ID No: 1 and the antagonist inhibits CEA-specific immune responses.

41. A pharmaceutical composition comprising the peptide according to claim 40 and a pharmaceutically acceptable carrier.

42. A method of inhibiting CEA-specific immune responses comprising administration of the peptide according to claim 40 in an amount effective to inhibit the CEA-specific immune responses.

43. The method according to claim 42 wherein cytotoxic T lymphocytes specific for CEA or epitopes thereof are inhibited.

44. A peptide-pulsed cell comprising an antigen presenting cell pulsed with a peptide according to any of claims 1 through 6.

45. The peptide-pulsed cell according to claim 44 wherein the antigen presenting cell is selected from the group consisting of dendritic cell, B lymphocyte, monocyte and macrophage.

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